Systemic Exposure to Oral Infections — a Cardiometabolic Risk

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“During the last few years the conviction has grown continually stronger, among physicians as well as dentists, that the human mouth, as a gathering place and incubator of diverse pathogenic germs, performs a most significant role in the production of various disorders of the body, and that many diseases whose origin is enveloped in mystery, if they could be traced to their source, would be found to have originated in the oral cavity."

Dr. W. D. Miller
The Human Mouth as a Focus of Infection
The Lancet 1891

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List of original publications

The present thesis is based on the following original publications, referred to in the text by their Roman numerals I-V.

I. Liljestrand JM, Gursoy UK, Hyvärinen K, Sorsa T, Suominen AL, Könönen E, Pussinen PJ. 2014. Combining salivary pathogen and serum antibody levels improves their diagnostic ability in detection of periodontitis. *Journal of Periodontology.* 85(1):123-31.

II. Liljestrand JM, Paju S, Pietiäinen M, Buhlin K, Persson GR, Nieminen MS, Sinisalo J, Mäntylä P, Pussinen PJ. Immunologic burden links periodontitis to acute coronary syndrome. *Submitted.*

III. Liljestrand JM, Paju S, Buhlin K, Persson GR, Sarna S, Nieminen MS, Sinisalo J, Mäntylä P, Pussinen PJ. 2017. Lipopolysaccharide, a possible molecular mediator between periodontitis and coronary artery disease. *Journal of Clinical Periodontology.* 44(8):784-792.

IV. Liljestrand JM, Mäntylä P, Paju S, Buhlin K, Kopra KA, Persson GR, Hernandez M, Nieminen MS, Sinisalo J, Tjäderhane L, Pussinen PJ. 2016. Association of endodontic lesions with coronary artery disease. *Journal of Dental Research.* 95(12):1358-1365.

V. Liljestrand JM, Havulinna AS, Paju S, Männistö S, Salomaa V, Pussinen PJ. 2015. Missing teeth predict incident cardiovascular events, diabetes, and death. *Journal of Dental Research.* 94(8):1055-62.

In addition, some unpublished data are presented.

Abbreviations

AAP	American Academy of Periodontology
ABL	Alveolar bone loss
ACS	Acute coronary syndrome
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
AP	Apical periodontitis
AUC	Area under the curve
BCa	Bootstrap confidence interval
BMI	Body mass index
BOP	Bleeding on probing
CAD	Coronary artery disease
CAL	Clinical attachment loss
CHD	Coronary heart disease
CGP	Chronic generalized periodontitis
CI	Confidence interval
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
EC	Endothelial cell
ECG	Electrocardiogram
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GWAS	Genome-wide association study
HDL	High-density lipoprotein
HOMD	Human oral microbiome database
HRP	Horseradish peroxidase
HSP	Heat shock protein
Ig	Immunoglobulin

IL	Interleukin
LAL	Limulus amebocyte lysate
LBP	Lipopolysaccharide binding protein
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MetS	Metabolic syndrome
MHC	Major histocompatibility complex
NF-κB	Nuclear factor κ B
NRI	Net reclassification improvement
OMV	Outer membrane vesicle
OR	Odds ratio
ox-LDL	Oxidized low-density lipoprotein
PAI	Periapical index
PBS	Phosphate buffered saline
PPD	Pocket probing depth
RCF	Root canal filling
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RR	Risk ratio
RT	Room temperature
SE	Standard error
SES	Socioeconomic status
SMC	Smooth muscle cell
SPSS™	Statistical Package for Social Sciences
SR-A	Scavenger receptor class A
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Tris	Tris(hydroxymethyl)aminomethane
VLDL	Very low density lipoprotein
qPCR	Quantitative polymerase chain reaction

Abstract

Cardiometabolic risk indicates a cluster of factors predisposing to cardiovascular diseases (CVDs) and diabetes mellitus. The main underlying mechanism for CVDs is atherosclerosis, which is the leading cause of death worldwide. Accumulating evidence states that chronic infections may contribute to the total cardiometabolic risk e.g. via an increased systemic inflammation. The oral cavity is a probable culprit for chronic inflammations, considering that periodontitis and apical periodontitis (AP) are often asymptomatic and among the world's most prevalent infectious diseases. If left untreated, the affected teeth might need to be extracted, and missing teeth crudely indicates past or present oral diseases. The general hypothesis of this thesis was that periodontitis and AP increase the risk for CVD events due to bacterial mediators, such as lipopolysaccharide (LPS), which is a membrane structure of gram-negative bacteria and a potent proinflammatory molecule. A humoral immune response towards periodontal pathogens might indicate an immunologically hyperresponsive phenotype susceptible for chronic inflammatory diseases or serve as a biomarker for the oral pathogen burden. The antibodies might also have direct proatherogenic effects via molecular mimicry.

Our aim was to investigate associations between oral infectious diseases, oral bacteria, along with their respective adaptive immune response, endotoxemia and CVD. More specifically, we aimed to study: I) how oral abundance of the periodontal pathogens *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Porphyromonas endodontalis*, *Tannerella forsythia*, *Campylobacter rectus*, *Prevotella intermedia* and their serum antibodies associate with each other, periodontitis and coronary artery disease (CAD); II) salivary and serum LPS activity in relation to periodontitis, subgingival pathogen burden and CAD; III) associations between AP and CAD; IV) associations between missing teeth and incident CVD, diabetes and death.

We utilized three observational cohorts, namely the Health 2000 survey, the Parogene study and the FINRISK 1997 study, yielding one longitudinal- and four cross-sectional articles presented in this thesis. Depending on the database, information was available from: clinical and radiographic oral examinations; subgingival bacterial determinations by DNA-DNA hybridization; salivary and serum LPS activity measurements with Limulus amebocyte lysate assay; angiographic determinations of coronary status and; record linkage with national registers. For this thesis project, the pre-existing data was

complemented with bacterial detection by quantitative polymerase chain reaction and serum antibody measurements by enzyme-linked immunosorbent assay.

Oral pathogen levels correlated with corresponding serum antibody levels, demonstrating that oral bacteria initiate a humoral immune response. The circulating antibodies represent a systemic exposure to past or present oral infections. Subjects with a history of periodontitis had an elevated antibody response to the selected bacteria, and subjects with active periodontitis had simultaneously elevated oral pathogen levels. A combination of oral pathogens and corresponding antibodies has biomarker potential in periodontal diagnostics, particularly in situations where clinical and radiographic oral evaluation is not possible, such as in epidemiologic surveys and at general medical practitioners' offices.

Especially in periodontitis patients, the subgingival abundance of several periodontal pathogens contributed significantly to salivary LPS levels, which in turn associated with endotoxemia. High serum LPS activity associated with stable CAD, showing that LPS is a potential mediator between periodontitis and CAD. An elevated humoral immune response towards periodontal pathogens indicated an increased risk for acute coronary syndrome (ACS). The immunologic burden might therefore represent another path of mediation, since some antibodies have shown to auto-react with host cells in a pro-atherogenic manner.

Information on missing teeth was additive to the established cardiometabolic risk profiles and associated with incident CVD, diabetes or death, independently of typical confounders. The number of missing teeth is a practical marker for crude evaluation of cardiometabolic risk and it has potential as a self-report variable in large surveys. AP might increase systemic inflammation via similar mechanisms as periodontitis. In our study, AP had a confounder independent association with CAD, especially ACS.

Oral infectious foci, both periodontal and periapical, should be thoroughly evaluated by routine, as they provoke an increased risk for chronic non-communicable diseases in addition to an impaired oral health. Special focus should be directed towards patients with other cardiometabolic risk factors. We have provided novel information on endotoxemia and immunologic burden to periodontal pathogens as potential mediators between these diseases.

1 Review of the literature

1.1 Structure of teeth and surrounding tissues

The complete human dentition is comprised of 32 teeth; eight incisors, four canines, eight premolars and twelve molars. The main components of the tooth are the enamel, dentin, pulp tissue and root cementum. The periodontium, i.e. the tooth supporting tissues are composed of four principal components: gingiva, periodontal ligament, root cementum and the alveolar bone.

The enamel is the outer layer, up to 2.5 mm thick, normally covering the visible part of the tooth crown. The enamel tissue is the most mineralized tissue in the human body and is therefore specialized to withstand a lifetime of wear and acidic insults. The dentin composes a major part of the tooth structure. Unlike enamel, the dentin is dynamic and continues to grow throughout the life by formation of secondary or tertiary dentin by odontoblasts. It does not contain innervation or vasculature. The dentin contains microscopic channels, dentinal tubules 1-3 µm in diameter, radiating from the dentinoenamel junction to the pulp (Ingle et al. 2008). The dentin is more susceptible to cavities than enamel because of its soft and porous structure. (Fejerskov & Kidd 2008)

The dental pulp is the central neurovascular component of the tooth, containing fibroblasts and active white blood cells. It extends from the tooth's cervical region, through the roots into the apical foramina. The central zone of the pulp incorporates vessels and nerves surrounded by fibroblasts embedded in the intercellular matrix and collagen fibers. The vasculature and innervation reach the coronal pulp via the apical foramen and root canals. (Baumann et al. 2010) The outermost layer of the pulp contains viable odontoblast. The pulp is responsible for providing the organic components of the tooth with moisture and nutrients and for conveying sensory information. The dental pulp is able to produce tertiary dentine in response to injury. (Gerli et al. 2010)

The cementum layer covers the root and connects the tooth to the alveolar bone via anchorage by collagen fibers of the periodontal ligament. The periodontal ligament is approximately 0.15-0.4 mm wide and serves supportive, nutritive, sensory and remodeling functions. The principal fibers of the

periodontal ligament are the alveolodental fibers, which function as the main shock absorber for receiving substantial compressive forces generated at chewing. (Newman et al. 2002)

The crown of the tooth is surrounded by free gingiva, which is free from attachment to the underlying bone, and by attached gingiva which is stabilized by supra-alveolar periodontal fibers. The epithelium of free gingiva is divided into junctional epithelium, sulcular epithelium and oral epithelium. The healthy crevice between the gingiva and tooth has a probing depth of 2-3mm and called gingival sulcus. At the bottom of the sulcus, junctional epithelium attaches directly onto the enamel layer with hemidesmosomes for approximately 1mm. In a healthy state, the junctional epithelium along with stabilizing gingivodental collagen fibers constitute the main barrier between the oral environment and the body. The vertical distance between the bottom of the sulcus and the alveolar ridge is called biological width, which is approximately 2mm long. (Newman et al. 2002)

1.2 Oral microbiota

The oral cavity represents a unique microbial habitat and it incorporates a higher phylogenetic diversity than any other location of the gastrointestinal tract (Stearns et al. 2011). The relationship between oral microbiota and host is dynamic, and influenced by many aspects of modern lifestyle, such as diet and tobacco consumption. In the healthy state, microbial communities partake in critical metabolic, physiological and immunological functions. (Kilian et al. 2016)

The Human Oral Microbiome Database (HOMD) is a body-site specific microbial resource, which continually collects information on the oral microbiota and links it with phenotypic, phylogenetic, bibliographical and clinical data. The oral cavity has a diverse microbiota with over 700 prokaryotic endogenous species identified to date (Chen et al. 2010). Of these, 54% have been officially named, 14% are cultivated but unnamed and 32% are known as uncultivable phylotypes. Only 280 species have been isolated in culture and the remaining have been validated with 16s rRNA gene-based cloning studies (Dewhirst et al. 2010). A study by Bik et al. (2010) characterized the composition of the oral microbiota of participants with healthy oral tissues (Bik et al. 2010). A total of 26 oral sites per subject were sampled and pooled, whereafter 16s rRNA sequencing was implemented to detect 247

operational taxonomic from the entire population (1% difference cut-off). A broad overview of the oral microbiome is illustrated in a pie chart (**Figure 1**), which illustrates the 15 orally detected phyla, their respective taxon counts (based on the HOMD database) and the relative abundance (%) of the most detected phyla, as reported by Bik et al (Bik et al. 2010). The relative abundance of bacterial species, documented in the HOMD database (Chen et al. 2010), have been reported earlier (Palmer 2014), and further depicted in a pie chart in a review by Costalonga et al. (2014) (Costalonga & Herzberg 2014).

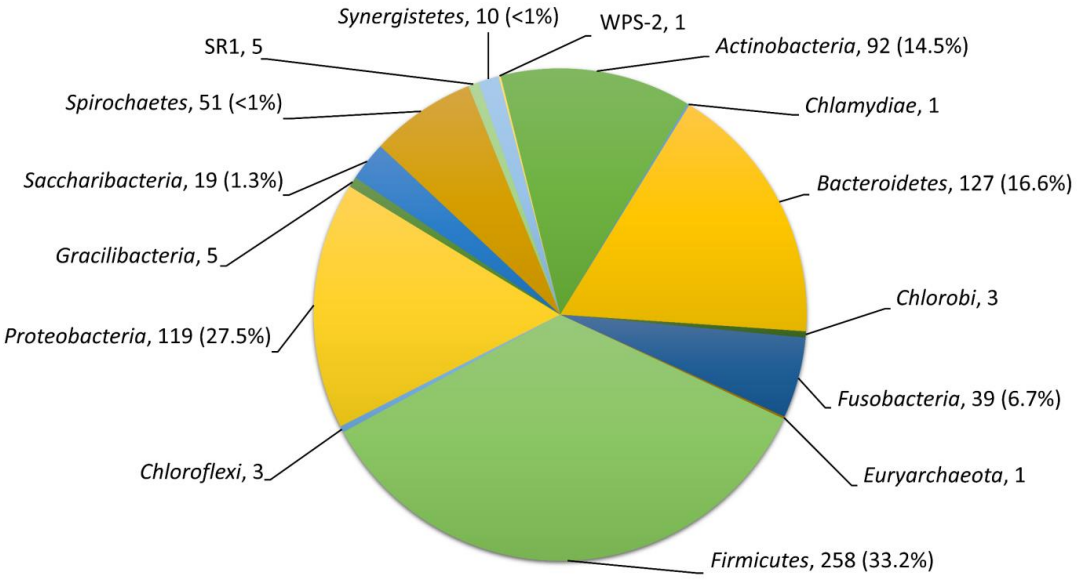

Figure 1. Orally detected phyla, taxon counts and relative abundance of phyla.

The detected phyla and their respective taxon counts are adapted from the Human Oral Microbiome Database (HOMD) (Chen et al. 2010). The size of the slice represents the proportion of phylum-associated taxon counts. To date, HOMD incorporates detailed information on 734 operational taxonomic units identified from 16S rRNA gene sequence analysis of oral isolates and clone libraries (similarity cut-off between phylotypes 98.5%). The cloning, sequencing, aligning, treeing and clustering methods used to create HOMD have been reported (Dewhirst et al. 2010). The relative composition (%) of the most abundant phyla are derived from a comprehensive, high resolution 16S rRNA sequencing study on 10 orally healthy individuals (similarity cut-off 99%) (Bik et al. 2010).

Next generation sequencing techniques provide an open-ended view over the whole breadth of the oral microbiome. However, new high throughput sequencing techniques, such as pyrosequencing, have already indicated that the oral microbiota might harbour over 19 000 phylotypes (Keijser et al. 2008). Of note, the vast majority of the newly identified taxa are present at very low levels, and the total impact of the species richness on oral health-disease equilibrium and physiology of the oral microbiota is yet to be determined (Keijser et al. 2008). It has been postulated that any individual harbours approximately 100-200 different bacterial species at a time, resulting in substantial diversity between people (Paster et al. 2006). However, instead of merely identifying oral species, more efforts should be made to study interactions, by community analyses based on systems ecology principles or metatranscriptomic studies for metabolic determinations, to truly understand forces that maintain stability or causes disease (Zaura 2012, Jorth et al. 2014).

The oral microbial composition differs depending on the site, especially between non-shedding hard tissue surfaces and continually shedding mucosal surfaces, which do not generally allow complex biofilm maturation. The relative abundance of bacterial genera in the oral cavity, saliva, dental plaque, and the periodontal pocket (both health and disease) have been reported earlier (Costalonga & Herzberg 2014). The development of salivary microbiota depends mainly on environmental factors and it remains remarkably stable over time (Stahringer et al. 2012). The relative bacterial composition in saliva has been shown to resemble most those of mucosal surfaces (Zaura et al. 2009), displaying higher proportions of e.g. *Streptococcus oralis*, *S. mitis* and *S. constellatus* compared to subgingival samples (Mager et al. 2003). A 16S rRNA sequencing study of salivary samples from geographically different locations showed, that compositional variation was prominent interindividually, but less dependent on geographic location. The genera *Streptococcus, Prevotella, Veillonella, Neisseria, Haemophilus*, *Rothia*, *Porphyromonas*, and *Fusobacterium* were widespread, frequently encountered in saliva and accounted for >70% of all detected sequences (Nasidze et al. 2009), and similar compositions of salivary microbiota has been reported earlier (Keijser et al. 2008). Even tough saliva imposes a smaller biodiversity compared to subgingival plaque, it is generally considered a "fingerprint" of the whole oral microbiota (Xu et al. 2015).

The oral cavity is a major gateway for microbes to enter the body, therefore one of the challenges in determinations of oral microbiota is separating exogenous transient species from true oral residents.

In health, the oral microbes co-exist in equilibrium as commensals, but might cause oral infectious diseases, such as tooth decay (caries), endodontic infections, periodontitis or pericoronitis if the environment is favourable for disease progression (Dewhirst et al. 2010). Oral infections have also been linked to various cardiometabolic disorders, such as cardiovascular disease (CVD), stroke, obesity and diabetes mellitus, and the role of microbiota in them is under intensive investigations worldwide (Seymour et al. 2007, Lockhart et al. 2012).

1.2.1 Microbial succession and biofilms

One of the hallmark features of oral microbiota is their capability to form biofilms, complex multispecies ecosystems in a structured and ordered manner. Biofilm formation is most pronounced on the non-shedding tooth surface, including the periodontal pocket. Some planktonic bacteria have an ability to adhere to the pellicle, a thin layer of proteins on the tooth surface. Secondary colonizing species co-aggregate with these early colonizers via receptors resulting in vivid cell-to-cell interactions. The microbes colonize their respective niches and alter the habitat, making it suitable for other species. Also the physical and chemical properties of the region or changes in the host causes alterations in the bacterial community. These phenomena's are called autogenic and allogenic microbial succession (Socransky & Haffajee 2005). The biofilm is an essential component involved in the development of caries and periodontal disease (Sanz et al. 2017).

The biofilm is beneficial for the bacterial community, while the co-adhesion along with an extracellular matrix provides structure and protection from the environment. Bacteria co-exist in symbiosis, where some species provide metabolites to others as energy sources and exchange genetic information. On the other hand, there is an active competition of living space, for example by secretion of bacteriocins, organic acids and different enzymes (Marsh & Zaura 2017). Various functions of the biofilm community are regulated by quorum sensing, a chemical communication between bacteria where gene expressions are regulated by cell density. The microenvironment of the biofilm is dynamic and enables succession towards capnophilic and anaerobic species (Hojo et al. 2009). Oral biofilms are generally non-pathogenic, but when the sensitive ecosystem turns out of balance by overload or weakened immune response, it might cause bacterial dysbiosis which

challenges health either locally or systemically (Hajishengallis & Lamont 2012, Arweiler & Netuschil 2016).

1.3 Oral inflammatory diseases

Dental caries is defined as a chronic slowly progressive disease at the tooth surface. It is caused by a disturbance in the physiological equilibrium in the dental biofilm, and may therefore be considered an infectious disease. Caries active and caries free individuals share approximately 50% of the supragingival plaque microbiome, and caries is frequently associated with the *Streptococcus, Veillonella* and *Actinomyces* species (Peterson et al. 2013). The gradual demineralization of tooth substance causes a localized destruction called cavities. The initial stages of caries are asymptomatic, and symptoms emerge when the disease progresses into dentin (Fejerskov & Kidd 2008). Untreated caries in permanent teeth is considered the most prevalent condition globally, affecting 2.4 billion people. The prevalence peaks at three age-groups; 6, 25 and 70 years of age (Kassebaum et al. 2015). In a national population-based survey of Finnish adults, the age-standardized prevalence of caries was 28% for men and 14% for women (Koskinen et al. 2012). An active caries lesion requires modification in the biofilm process to acquire lesion arrest, which might be achieved by plaque control, fluoride, dietary control or salivary stimulation. Operative treatment, i.e. placing a restoration, is often required in lesions extending to the dentin to facilitate plaque control (Fejerskov & Kidd 2008). If active caries is left untreated, it will progress into the pulp chamber causing an endodontic infection. The tissue is gradually infected and devitalized, and oral microbes progress into the root canals. In such cases, a chronic inflammatory reaction develops around the apical foramen, which is believed to prevent spread of infection into the periapical tissues (**Figure 2**) (Ingle et al. 2008).

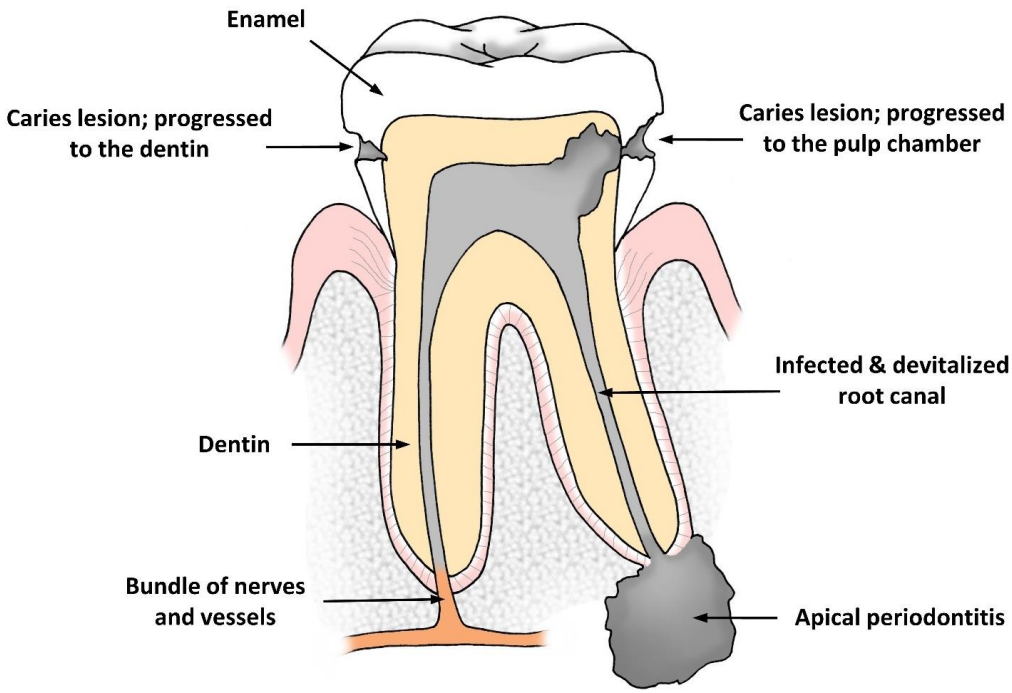

Figure 2. Anatomy of the infected tooth.

These endodontic lesions, also called apical periodontitis (AP), are very common; the prevalence of having ≥1 periapical lesions has been reported to be up to 61% (Jimenez-Pinzon et al. 2004) and approximately 5% of all teeth have been reported to have radiographically evident periapical lesions (Pak et al. 2012). For the sake of clarity the term "apical periodontitis" (AP) will be applied throughout this thesis. An endodontically infected tooth is treated either by chemomechanical debridement of the root canal system, i.e. endodontic treatment, or alternatively by extraction of the tooth (Ingle et al. 2008).

Marginal periodontitis, commonly referred to simply as periodontitis, is a chronic inflammatory disease in the tooth supporting tissues – that is, the periodontium (**Figure 3**). It is a polymicrobial infectious disease with several proposed pathogens, and the resulting tissue destruction is caused by an interplay with bacterial activity and aggressive host response (Darveau 2010). Along with dental

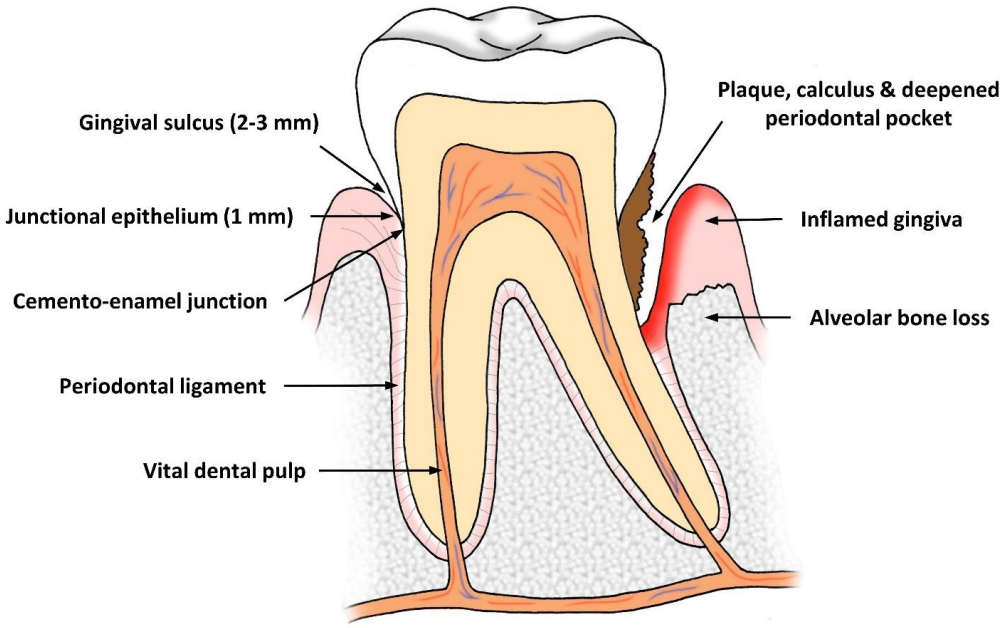

Figure 3. Periodontal anatomy in health and disease.

Left side represents a healthy state. Right side represents marginal periodontitis.

caries, periodontitis is one of the most common infectious diseases, with an age-standardized prevalence of 11% for its severe form (Kassebaum et al. 2014), making it the sixth most common disease globally (Frencken et al. 2017). Approximately two thirds of the Finnish adult population have ≥4mm deep periodontal pockets, which is an indicator for mild periodontal disease (Koskinen et al. 2012). Periodontitis and the associated tissue destruction leads to increased tooth mobility, and eventually to tooth loss if left untreated (Newman et al. 2002).

1.4 Marginal periodontitis

A reclassification of plaque induced periodontal diseases were developed at the 1999 International Workshop for Classification of Periodontal Diseases and Conditions, including seven types of

periodontal diseases; 1) gingivitis, 2) chronic periodontitis, 3) aggressive periodontitis, 4) periodontitis as a manifestation of systemic diseases, 5) necrotizing periodontal diseases, 6) abscesses of the periodontium and 7) periodontitis associated with endodontic lesions (Armitage 1999). The American Dental Association and The American Academy of Periodontology (AAP) has developed a more clinical perspective for classifying marginal periodontitis in four case type definitions: 1) gingivitis, 2) early periodontitis, 3) moderate periodontitis and 4) advanced periodontitis (Armitage 2003). The AAP has reported up to 10 different classification systems for periodontal disease in the past 20 years (Newman et al. 2002), and the research field of periodontal diagnostics is plagued by significant heterogeneity of methodology and diagnostic criteria. Consequently, no international standard is accepted to date (Page & Eke 2007, Albandar 2007, Savage et al. 2009).

1.4.1 Diagnostics

Marginal periodontitis is always preceded by gingivitis. Gingival inflammation is characterized by redness, swelling, suppuration and bleeding on probing (BOP). A threshold of 15-30% of bleeding sites after probing is often considered as a clinical marker for gingivitis (Badersten et al. 1990, Joss et al. 1994, Sanz et al. 2015), and is also an important marker for active periodontitis. Periodontitis always involves tissue destruction and alveolar bone loss (ABL) (Newman et al. 2002). The tissue destruction is evaluated by measurements of pocket probing depth (PPD), gingival recession, increased tooth mobility, furcation lesions, and radiographically evident ABL. The periodontal examinations should include a full mouth probing of 6 sites per tooth to be reliable, even though partial mouth examinations are frequently used in surveys (Kingman et al. 2008). A PPD of ≥4 is generally considered pathological (Newman et al. 2002).

Clinical attachment loss (CAL) is defined as the distance between the cemento-enamel junction and the bottom of the gingival pocket. CAL is considered the golden standard for evaluating periodontal attachment loss, (Armitage 2003) but is less used in clinical practice due to its time consuming nature (Page & Eke 2007). The clinically and radiologically determined parameters ought to be combined with other factors, such as patient age, past dental history, systemic conditions, medications and smoking to acquire the final diagnosis. State of the art diagnosis of periodontitis is usually not possible

in large surveys, and a variety of less rigorous definitions have commonly been used (Spangler et al. 2012). The Centers for Disease Control and Prevention and the AAP have proposed case definitions for detection of periodontitis in population-based surveys, which are solely based on CAL and PPD. In that definition, one site with PPD ≥5mm indicates mild periodontitis and ≥2 interproximal sites with PPD ≥5mm indicates moderate periodontitis (Eke et al. 2012). Adapted threshold levels PPD ≥4mm and PPD ≥6mm have also been used in population based surveys as disease indicators (Suominen-Taipale et al. 2008).

A list of commonly used periodontal markers, their abbreviations and indications

Bleeding on probing	BOP	Indicates active gingival inflammation
Pocket probing depth	PPD	Indicates a history connective tissue destruction or gingival swelling
Clinical attachment loss	CAL	Indicates a history of connective tissue destruction with accompanying alveolar bone destruction (clinical parameter)
Alveolar bone loss	ABL	indicates a history of tissue destruction, specifically alveolar bone destruction (radiographic parameter)

Supplemental diagnostic tests might be useful in screening of periodontitis patients or determining disease activity. They can be used to detect substances associated with putative periodontal pathogens, host derived enzymes, tissue breakdown products or inflammatory mediators. This information is gathered by collection of saliva, gingival crevicular fluid, or subgingival microbial samples (Armitage 2003). Microbial cultures are useful in some cases for selection of antimicrobial therapy. Point of care tests have been developed for determining enzymes with tissue breakdown capabilities from oral fluids, such as saliva (Rathnayake et al. 2017).

Vigorous research is warranted for detection of periodontitis-associated biomarkers to aid epidemiologic surveys and development of personalized risk assessment tools (Ebersole et al. 2015). Saliva is an intriguing media for diagnostics, while it is non-invasive and easily collected (Liu & Duan 2012) and some bacterial or host derived salivary markers have been proposed to be useful especially

in large population surveys where oral examinations are not feasible (Gursoy et al. 2011). However, mere microbial determinations from saliva have not been successful in periodontal diagnostics, while there is a high degree of intra-individual variations in the microbiota and saliva incorporates microbes also from healthy sites (Könönen et al. 2007, Hyvärinen et al. 2009, Mira et al. 2017). Serum biomarkers, especially antibody production towards periodontal pathogens, have also been proposed to be moderately accurate in classification of periodontal status (Dye et al. 2009, Pussinen et al. 2011). However, most of the proposed tests are yet to be verified and are not routinely used in clinic for periodontal diagnostics (Armitage 2003).

1.4.2 Etiology and microbiology

It has been long known that accumulation of dental plaque and microbial succession from a dominance of gram-positive aerobes towards gram-negative anaerobic species causes gingivitis, and that the gingival inflammation is reduced by removal of the biofilm (Theilade et al. 1966). The microbial shift towards a more pathogenic community leads to tissue destruction, partly by direct bacterial virulence mechanisms and mostly due to a disruption in the expression of host inflammatory mediators **(Figure 4)** (Khan et al. 2015). The gingival biofilm is in close contact with the porous epithelium, and the periodontium copes with constant microbial stimulation with a highly-orchestrated expression of innate host defense mechanisms. Together with regenerative and biomechanical signaling systems, the innate defense mechanisms keep the tissue in homeostasis and provides sufficient microbial protection by recruitment of leukocytes, primarily polymorphonuclear neutrophils (Darveau 2010). Stressors applied to the symbiotic state can perturb the homeostasis into a state where the microbial population is significantly altered. These stressors may include alterations in the immune response, for example impaired neutrophil function, and activities of keystone pathogen species (Sanz et al. 2017). The microbial succession is largely guided by micro-

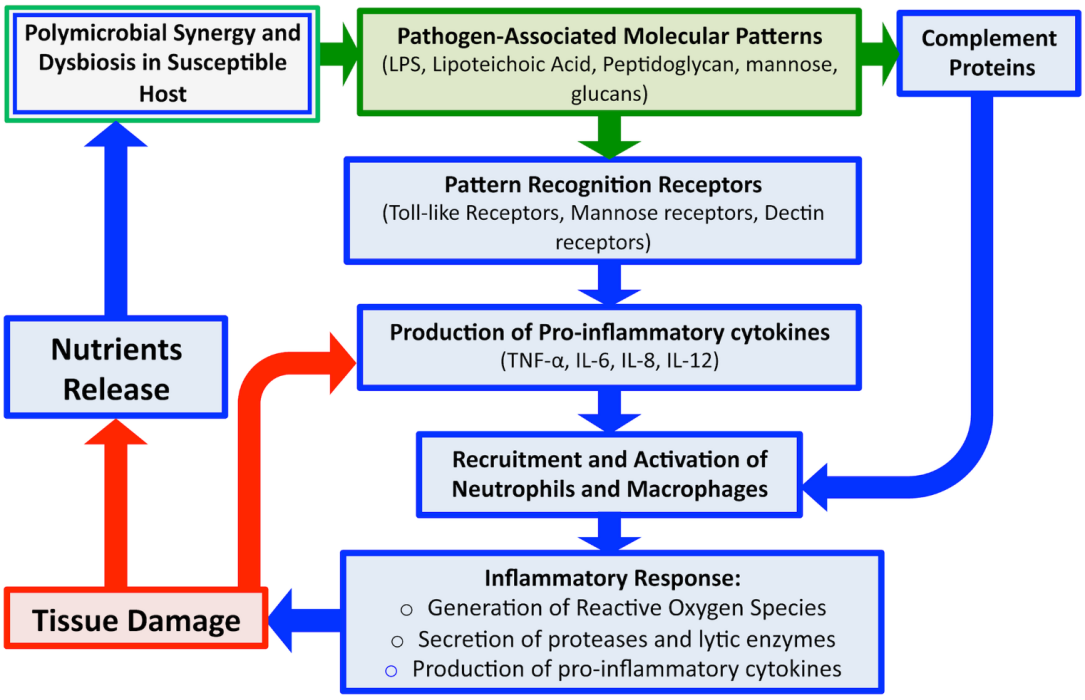

Figure 4. Pathogenesis of periodontitis.

A complex interplay of pathogenic microbes (green) and host derived factors (blue) are responsible for the disease-associated tissue destruction (Khan et al. 2015). Reprinted with permission from Public Library of Science (PLOS).

environmental factors; “inflammo-philic” species are favored by tolerance to such alterations in pH, temperature and availability of fermentable carbohydrates and host proteins (Marsh & Zaura 2017). The tissue destruction in periodontitis is largely due to a disruption of tissue homeostasis, by bacterial by-products and subsequent upregulation of pro-inflammatory mediators, resulting in increased levels of tissue destructive enzymes and enhanced bone loss by osteoclast activation (Darveau 2010). Of note, there is a significant intra-individual variation in the oral microbial composition depending on if the site of interest represents a diseased periodontal pocket or supragingival plaque (Mira et al. 2017).

In the late 1970s, it was noted that periodontitis patients had dramatic compositional changes in the microbiota compared to healthy controls. Consequently, the focus in periodontal etiology was shifted

towards putative periodontal pathogens (Socransky 1977). The color coded bacterial complexes were introduced by Socransky et al. in 1998, in which they stated that some species are highly associated with each other and with clinical disease (Socransky et al. 1998). This concept is still much used today in periodontal research and the periodontitis-associated species are used as biomarkers of the dysbiotic biofilm.

The color-coded complexes have provided a framework for describing and understanding the subgingival ecosystem (Socransky et al. 1998, Socransky & Haffajee 2005). Socransky et al. used clustering and community ordination techniques for identifying species that were highly associated with each other in the oral biofilm and the acquired complexes could be considered as a simplified reflection of microbial succession. The yellow, purple and green complexes represent early colonizers promoting the more pathogenic orange complex, which in turn facilitates red-complex colonization. The red-complex was highly correlated with parameters indicating periodontal disease, such as PPD and BOP (Socransky et al. 1998).

Orange and red bacterial complexes

Orange complex:	*Prevotella intermedia, Prevotella nigrescens, Parvimonas micra, Fusobacterium nucleatum (subsp. vincentii, nucleatum, polymorphum, periodonticum)*
Red complex:	*Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*

Aggregatibacter actinomycetemcomitans has been stated to be one of the main etiological species in periodontitis, along with the red complex species (Sanz et al. 2017). *A. actinomycetemcomitans* (serotype a) is a member of the green complex (Socransky et al. 1998). It is a facultatively anaerobic, non-motile, non-sporing, small gram-negative rod and a member of the indigenous oral microbial flora. This bacterium can cause significant alterations in its host because of its powerful toxins and its ability to adhere, invade and transmigrate host cells (Henderson et al. 2010). It has been traditionally linked to localized juvenile periodontitis (Christersson 1993), although its essential role in chronic marginal periodontitis has been established (Henderson et al. 2010).

As the understanding of microbiology in the periodontal etiology evolved, the role of environmental factors was stressed in the "ecological plaque hypothesis" in 2003 (Marsh 2003). The disruption of host homeostasis by a complex inflammatory response for a polymicrobial interaction was acknowledged in 2010, although red complex species were still considered key species in the progression of marginal periodontitis (Darveau 2010). Chronic periodontitis is surely not a monoinfection in any individual, while dysbiosis in periodontitis is associated with an increase in bacterial diversity (Sanz et al. 2017). Recently, Hajishengallis et al. introduced the concept that host modulation is resulted by colonization of "keystone pathogens", with a focus on *P. gingivalis* (Hajishengallis et al. 2011). They have also promoted a theory of "polymicrobial synergy and dysbiosis", in which they state that keystone pathogens might disproportionately elevate the virulence of the entire proinflammatory microbial community resulting in the destructive host response (Hajishengallis & Lamont 2012). These new hypotheses are in line with the modern way of thinking, that oral microbiota in health and disease should be evaluated by a series of functions and interactions. Mere listing of pathogens present is hardly sufficient while they elicit different responses inter-individually (Lloyd-Price et al. 2016). Even though the microbial etiology of periodontitis is ever changing, bacteria undeniably play a major part and new "periodontal pathogens" are suggested as research develops with modern methodology (Perez-Chaparro et al. 2014).

1.4.3 Disseminating effects of periodontitis

The first evidence of oral microbes being able to invade host cells was reported by Meyer et al. in 1991 (Meyer et al. 1991). To date, there is ample evidence for host invasion by several periodontal pathogens and it has been postulated that this represents a sophisticated evasion strategy for countering host defense. In fact, intracellular invasion provides a nutritional niche and protection from certain immune responses (Reyes et al. 2013). Furthermore, bacteria are known to enter the systemic circulation, especially during dental procedures, but also during daily mastication and brushing of teeth (Olsen 2008). As patients with periodontitis have 8-20 cm^2 surface of ulcerated subgingival epithelium (Hujoel et al. 2001b), they are more prone for transient bacteremia in their daily routines (Forner et al. 2006) and dental procedures (Horliana et al. 2014). Many gram-negative

bacteria are known to shed outer membrane vesicles (OMVs), which contains several virulence factors, such as lipopolysaccharide (LPS), proteases and adhesins (Miyakawa et al. 2004). For instance, *P. gingivalis* OMVs have shown to possess platelet aggregation-inducing activity *in vitro* (Sharma et al. 2000). Thus, not only whole bacteria, but also their by-products are dispersed into the systemic circulation. Oral bacteria have been frequently detected in extra-oral sites, although most used detection methods do not discriminate living from dead bacterial cells (Reyes et al. 2013). However, viable periodontal pathogens have been detected in atherosclerotic plaque samples also, providing evidence for systemic invasion and supporting the hypothesis that oral bacteria play a part in atherogenesis (Kozarov et al. 2005).

Pathogens that are frequently detected in extra-oral sites, represent typically established periodontal pathogens, such as *A. actinomycetemcomitans, F. nucleatum, P. gingivalis, P. intermedia*, and *T. forsythia* (Han & Wang 2013). Many such studies are biased by initial hypothesis, restricting found species to those which are studied. However, hypothesis-free pyrosequencing and 16s rRNA studies with GenBank data alignment have confirmed frequent detection of oral species in endarterectomy specimens (Koren et al. 2011, Calandrini et al. 2014). Moreover, oral microbes have been detected in extra-oral sites associated with adverse pregnancy outcomes (amniotic fluid, fetal membranes, cord blood, neonatal gastric aspirates, and fetal lung and stomach), rheumatoid arthritis (synovial fluid), inflammatory bowel disease (mucosal samples), respiratory tract infections (tracheobronchial isolates) and organ abscesses (brain, lung, liver and splenic abscesses). However, further studies are required to determine what role these bacteria have in extra-oral infections. (Han & Wang 2013) Infective endocarditis is a common life-threatening infection syndrome (Baddour et al. 2015). It is frequently associated with orally originated bacteria, mainly *streptococcus* and *staphylococcus* species (Parahitiyawa et al. 2009). These common oral bacteria mainly reside in the supragingival plaque and take part in the etiology of caries and endodontic infections, rather than periodontitis (Lockhart et al. 2009).

The oral microbiota has been shown to have systemic effects also by promoting a local proinflammatory response, causing an elevated systemic inflammation. Inflammatory mediators are likely to be easily distributed in patient with periodontitis, due to increased vasculature and vascular diameter in the inflamed periodontium (Zoellner & Hunter 1991). There is substantial evidence that

the tissue adjacent to periodontal lesions have a high concentration of proinflammatory cytokines, eicosanoids and other destructive mediators such as matrix metalloproteinases, a group of proteolytic enzymes (Preshaw & Taylor 2011).

It is commonly thought that some of these proinflammatory mediators are dispersed into the circulation (Schenkein & Loos 2013). Oral infections also promote systemic inflammation by increased hepatic production of proinflammatory mediators. Patients with periodontitis have been reported to have elevated levels of plasma acute phase proteins (CRP), fibrinogen, interleukins (IL)-6 and -18 compared to periodontally healthy controls (Loos et al. 2000, Leivadaros et al. 2005, Paraskevas et al. 2008, Buhlin et al. 2009, Higashi et al. 2009, Fedele et al. 2011) and periodontal therapy has shown to reduce systemic levels of these proinflammatory molecules (Mattila et al. 2002, Paraskevas et al. 2008, Higashi et al. 2009). Intensive treatment of periodontitis has also shown to improve flow-mediated dilation, a common measure for endothelial function, in a 6-month follow-up (Tonetti et al. 2007), and poor response to periodontal treatment has indicated increased risk for CVD-incidence (Holmlund et al. 2017b). However, the inflammatory markers are usually only slightly increased in periodontitis patients, and the beneficial effects of periodontal therapy on inflammatory markers are inconclusive (Teles & Wang 2011). Still, chronic low-grade inflammation, caused by oral inflammations, has potential to exacerbate inflammatory processes in other organ systems, adding the risk for several chronic non-communicable diseases (Loos 2005).

1.5 Apical periodontitis

AP, commonly called endodontic lesion, is an inflammatory reaction at the apex of an infected non-vital tooth. Its main function is to prevent spread of bacterial infection into the surrounding alveolar bone (Ingle et al. 2008). An endodontically treated tooth is easily detectable in radiographic images, due to high contrast of root canal fillings (RCFs). It is noteworthy, that up to 78% of endodontically treated teeth have RCFs of inadequate quality, and approximately 36% of them have radiographically evident AP (Pak et al. 2012). This finding demonstrates the high rate of persistent or recurrent endodontic infections.

1.5.1 Diagnostics

In clinic, AP is diagnosed by combining clinical examinations with radiographic imaging. The infected tooth does not usually respond to vitality tests, as is the case with previously root canal treated teeth. Symptomatic (acute) AP is characterized by tenderness to percussion, and asymptomatic (chronic) AP is mainly detected as a radiographically evident radiolucent area at the tip of the root, although inflammation caused by long standing pulp pathosis might result in radio-opaque condensing osteitis (Gutmann et al. 2009). Acute periapical abscesses associates with spontaneous pain, tenderness to pressure, pus formation and eventual swelling of adjacent tissues. (Ingle et al. 2008)

In endodontic research, AP is mainly diagnosed with radiographic imaging. One of the most commonly used radiographic imaging techniques in surveys is panoramic tomographies, explicitly, while they have proven to have low radiations doses, costs and specific field of interest (Gomes et al. 2015). Even slight radiographically evident widening of the periapical space is associated with a diseased tooth, either being a symptomatic tooth with irreversible pulpitis or precursors for an established AP in a necrotic tooth (Carrotte 2004). A frequently used scoring method for endodontic pathosis is the periapical index (PAI), introduced by Ørstavik et al. (1986). This 5-staged scoring method has shown to be reasonably accurate, reproducible and able to discriminate between subpopulations (Ørstavik et al. 1986).

1.5.2 Etiology and Microbiology

Endodontic infection, predominated by gram-negative anaerobic species, activates the periapical complement cascade because of inter alia high LPS concentrations (Graunaite et al. 2012). The pathogenesis of marginal and apical periodontitis shares common characteristics, both being infection induced chronic inflammatory diseases (**Figure 4**). The resulting inflammatory reaction upregulates the endothelial expression of intercellular attachment molecule 1 and increases local concentrations of the chemotactic complement factor C5a. This promotes margination of polymorphonuclear leukocytes, mainly neutrophils, into the affected area. The inflammation cascade, caused by pathogen or host derived proinflammatory cytokines (e.g. IL 1, -6, -8, interferon-ɣ, tumor necrosis factor [TNF]-

α), complement activation, LPS and chemokines causes hyperemia, vascular congestion, edema, stimulation of leukocytes, activation of the production of prostaglandins and proteolytic enzymes and promotes bone resorption (Graunaite et al. 2012). Usually the resulting periapical granuloma and host-pathogen interactions associated with it reaches equilibrium, but a homeostatic imbalance might promote abscess and sinus tract formation (Ingle et al. 2008).

Endodontic infections are polymicrobial with a high degree of inter-individual variability. More than 400 bacterial species have been found in infected root canals with anaerobic culturing and culture-independent molecular biology methods (Siqueira & Rôças 2009b). A mean species richness of 10-20 phylotypes are usually found in any infected root canal (Siqueira & Rôças 2009b). The diversity of endodontic microbiota in various disease phenotypes was evaluated in a comprehensive review by Siqueira and Rôças (2009) (Siqueira & Rôças 2009c). The main associated phyla are *Firmicutes* (e.g. *P. micra*), *Actinobacteria*, *Synergistes*, *Spirochaetes*, *Fusobacteria* (e.g. *F. nucleatum*), *Proteobacteria* (e.g. *Campylobacter rectus*), *TM7*, *SR1* and *Bacteroidetes* (e.g. *T. forsythia*, *P. gingivalis, Porphyromonas endodontalis* and *Prevotella spp*.). The phyla *Bacteroidetes* and *Firmicutes* are believed to be the most abundant in endodontic infections (Siqueira & Rôças 2009c, Hong et al. 2013, Tzanetakis et al. 2015). Some species are highly correlated with primary endodontic infections with moderate-strong degree of evidence, and are thus considered true etiological pathogens; *Filifactor alocis, Pseudoramibacter alactolyticus*, *P. micra*, *Dialister invisus*, *Dialister pneumosintes*, *F. nucleatum*, *T. denticola*, *P. endodontalis*, *P. gingivalis*, *T. forsythia*, *Bacteroidetes oral clone X083*, *Prevotella baroniae*, *P. intermedia* and *Prevotella nigrescens* (Siqueira & Rôças 2009c). These might represent keystone species maintaining the stability and virulence of a microbial community (Siqueira & Rôças 2009b). Of note, several of these species are also considered etiological pathogens for marginal periodontitis.

Pyrosequencing techniques have been used to evaluate if the phylogenetic diversity is different between primary and persistent endodontic infections, with ambivalent results (Hong et al. 2013, Tzanetakis et al. 2015). Much like with marginal periodontitis, a holistic view on pathogenic bacterial biofilm community has emerged, where any partaking species is considered etiologically relevant to AP (Siqueira & Rôças 2009a). Due to difficulties in characterizing the role of individual species in AP,

the only confident statement about microbial etiology in endodontic infections is that they are poly-microbial (Aw 2016).

1.5.3 Disseminating effects of apical periodontitis

As AP shares several characteristics with periodontitis, including microbial community and composition of inflammatory mediators, it is likely that they share similar systematic effects (Silva et al. 2007). In contrast to periodontal infections, no epithelial barrier is found between the necrotic infected root canal and the highly vascular granulomatous tissue in periapical infections. Therefore, dissemination of bacteria or their by-product is likely and supported by the finding that 31-54% of root canal treatments result in detectable levels of endodontically originated bacteremia, when measured during treatment and 10 minutes afterwards (Olsen 2008).

Levels of circulating inflammatory markers in patients with AP was evaluated in a systematic review and meta-analysis by Gomes et al. in 2013. They concluded that individuals with AP had rather consistently elevated serum levels of CRP, IL-1, IL-2, IL-6, total immunoglobulin (Ig)-A, IgG, and IgM, together increasing a systemic low-grade inflammation burden (Gomes et al. 2013). More recently, it has been found that AP might elevate systemic levels of TNF-α receptors (Singhal & Rai 2017), reactive oxygen species (ROS) (Inchingolo et al. 2013), fibrinogen (Vidal et al. 2016) or glycated hemoglobin HbA1c (Sanchez-Dominguez et al. 2015). The proposed elevation of systemic inflammatory markers by AP has been confirmed in murine models (Cintra et al. 2016).

1.6 Missing teeth

Caries and periodontitis are the two most important reasons for tooth loss worldwide. In general, caries is the leading cause of tooth extractions at the individual level, and periodontitis is the main reason at tooth level (Phipps & Stevens 1995, Richards et al. 2005). Especially in the older population, the relative contribution of periodontitis increases (Reich & Hiller 1993, Agerholm 2001, Gomes et al. 2012b). However, one should bear in mind that the number of missing teeth is also affected by other

factors, such as unerupted third molars, congenitally missing teeth, trauma, impacted teeth or extractions due to orthodontic indications.

1.7 LPS

LPS, or endotoxin, is a membrane structure of most gram-negative bacteria (Munford 2016). It plays an important role in the integrity of the outer-membrane permeability barrier and participates extensively in the host-pathogen interplay. The LPS molecule consists of a highly-conserved lipid-A component, which is usually glycosylated with a core oligosaccharide and further with a hypervariable O-antigenic polysaccharide. The LPS structures are modified to promote survival of bacteria in their respective niches (Whitfield & Trent 2014). LPS is a potent proinflammatory mediator both locally and systematically, as it promotes production of cytokines, lipid mediators and ROS. The resulting proinflammatory response is considered a classic trigger for septic shock. (Cohen 2002)

Systemic LPS is termed endotoxemia, and its principal effects are mediated by complex formation with LPS-binding protein (LBP) and recognition of the lipid-A region by the plasma membrane protein toll-like receptor (TLR) 4 (Raetz & Whitfield 2002). TLR:s are a family of pattern recognition receptors expressed on inter alia immune, vascular and myocardial cells. They detect pathogen-associated molecular patterns, such as LPS, and play an important part in the innate immunity by regulating transcription via nuclear factor κ B (NF-κB) signalling (Frantz et al. 2007). Largely due to these mechanisms, endotoxemia has been shown to associate with atherosclerosis, obesity, type 2 diabetes, CVD and non-alcoholic fatty liver disease (Piya et al. 2013). Endotoxemia is also known to shift the homeostatic balance between procoagulant and anticoagulant mechanisms resulting in a net effect favouring clot formation (Cohen 2002).

Extravascular LPS is transferred to the systemic circulation through several proposed pathways. Clinically significant amounts of circulating LPS is directly released by blood borne bacteremia (Brandtzaeg et al. 1992). However, a majority of circulating endotoxin is probably translocated from the small intestine. This transmucosal transport is promoted by a high-fat diet and metabolic diseases such as diabetes (Piya et al. 2013). The intestinal endothelial cells release LPS via triglyceride rich lipids, such as chylomicrons (Ghoshal et al. 2009), and LPS is transferred to systemic circulation via

lymphatics (Lu & Munford 2011). The transport of LPS, which is an amphipathic molecule, is facilitated by binding to carrier proteins or lipoproteins. These transfer proteins, such as phospholipid transfer protein, LBP and bactericidal/permeability-increasing protein, have a partly protective effect by neutralization of LPS (Hailman et al. 1996).

Approximately 80-97% of circulating LPS is bound to lipoproteins, including all major lipoprotein classes, but mostly high-density lipoprotein (HDL) due to most efficient LPS-scavenging capabilities. (Levels et al. 2001, Harris et al. 2002). Lipoproteins constitute an endogenous LPS-detoxification system by delivery of LPS to biliary secretion (Levels et al. 2001). Also, the rapid binding to lipoproteins have shown to attenuate the proinflammatory effects of LPS (Harris et al. 2002). In patients with periodontitis, a large fraction of LPS has been shown to associate with pro-atherogenic lower density lipoproteins (Kallio et al. 2008) similarly as in sepsis (Levels et al. 2003) and in mice infected with *Aggregatibacter actinomycetemcomitans* (Tuomainen et al. 2008).

The oral environment changes in patients with oral infections; the microbiota favors gram-negative anaerobes (Darveau 2010), many of which have an invasive nature (Han & Wang 2013), and even gentle mastication has been shown to cause endotoxemia (Geerts et al. 2002) and bacteremia (Forner et al. 2006). Considering the facts that bacteria are abundant in saliva (~10^9 CFU/ml), 1 liter of saliva is swallowed daily and that LPS retains its biologic activity in acidic conditions, it seems reasonable to claim that the oral microbiota is a potential source of endotoxemia (Erridge 2011). This notion is supported by the observation that patients with periodontitis have higher salivary concentrations of TLR-stimulants than periodontally healthy controls (Lappin et al. 2011). Furthermore, elevated serum LPS levels have been detected in periodontitis patients and periodontal treatment might lower LPS, among other pro-atherogenic functions (Pussinen et al. 2004b). No previous studies have simultaneously characterized the oral microbiota, salivary and serum LPS-levels and periodontitis.

1.8 Adaptive immunity

The adaptive immune system, also called acquired immune system, eliminates pathogens or prevents their growth with a set of specialized cells. In this context, we only refer to the humoral part of adaptive immunity. In short, non-self antigens are internalized and presented by antigen presenting cells, such as dendritic cells, B-lymphocytes and macrophages. The periodontium harbors mainly major histocompatibility complex (MHC) II expressing antigen presenting cells, which are recognized by T-lymphocytes and delivered to lymph nodes for activation and differentiation (Newman et al. 2002). Complex interactions with effector molecules result in cytotoxic T-lymphocytes and antibody producing B-lymphocytes, specific for the encountered antigen, constituting the hallmark for adaptive immunity. Lymphocytes can form memory cells, which are rapidly activated on secondary exposure. Hence, the immune system has "adapted" towards a stronger and faster immunity regarding a specific antigen (Delves et al. 2011).

Antibodies, or immunoglobulins (Igs), are "Y"-shaped globular glycoproteins that bind to their respective antigen and support the immune response by agglutination, priming phagocytosis and stimulating other immune reactions, such as complement activation. Five distinct classes of Igs have been detected in humans; IgG, IgA, IgM, IgD and IgE. They trigger different effector responses due to structural heterogeneity in their Fc region. Circulating IgG and IgE are generally monomeric, whereas IgM occurs as a pentamer. IgA occurs as a monomer in serum and as a dimer in secretions, such as saliva (Delves et al. 2011).

1.8.1 Adaptive immunity in oral infectious diseases

As previously discussed, oral bacteria are in intimate contact with the surrounding tissues. Therefore, specific antibodies against oral bacteria are produced both in healthy and diseased individuals. The population of leukocytes in periodontal tissues are predominated by T-cells in gingivitis and stable lesions, but in patients with active established periodontitis the dominance is shifted towards B-cells and plasma cells. Due to local production, higher levels of antibodies towards periodontal pathogens appear in the gingival crevicular fluid compared to serum (Ebersole et al. 2013). Serum antibodies

produced against periodontal pathogens are primarily IgG-type, while IgA and IgM are produces to a lesser degree. Several animal models indicate that high antibody titers against periodontal pathogens have a protective effect on periodontitis, and even development of vaccines has been proposed for periodontitis (Persson 2005). The proposed relationship is probably more complicated, considering that successful antibody response might provide selective advantage for opportunistic pathogens to remain in the chronic colonization of subgingival ecology and that complex multispecies biofilms and cell invasion provides evasion strategies for the bacteria (Ebersole et al. 2013). Therefore, it is not entirely clear in which proportion antibodies towards periodontal pathogens have a protective effect or if they participate in the pathogenesis of periodontitis and other chronic non-communicable diseases (Newman et al. 2002).

According to the consensus report of the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions, patients with localized aggressive periodontitis show robust antibody responses to infecting agents, while in contrast, patients with generalized aggressive periodontitis show poor systemic antibody responses (Lang et al. 1999). This statement has been criticized, while it was largely based on the hypothesis that antibodies have merely a protective role, and contradictory evidence have emerged (Hwang et al. 2014).

Elevated systemic antibody levels for periodontal bacteria have been found in patients with chronic marginal periodontitis (Ebersole et al. 1987, Papapanou et al. 2000, Dye et al. 2009, Kudo et al. 2012, Buhlin et al. 2015), but similar evidence is not available regarding AP (Gomes et al. 2013). However, this correlation is rather influenced by demographic, behavioral, and oral- and general health-related characteristics (Vlachojannis et al. 2010). For example, the age of the subject constitutes an interesting confounding factor. It has been suggested that young patients have milder systemic antibody response towards oral bacteria, probably due to shorter duration of bacterial exposure and less of the total bacterial load (Bimstein & Ebersole 1991). On the other hand, older age groups might have less humoral immune response against oral bacteria (Papapanou et al. 2000, Vlachojannis et al. 2010), possibly due to age related immunosenescence (McArthur 1998). Therefore, the immune response might be best predictable in adults, excluding youth and seniors.

Of note, the prevalence or levels of periodontal bacteria are stronger determinants for antibody production than periodontal diagnosis (Pussinen et al. 2011). The antibody response might remain elevated for some time, even after treatment of disease and supposed reduction of bacterial load (Buhlin et al. 2015), especially plasma IgG levels against periodontal pathogens have been shown to be extremely stable for up to 15 years (Papapanou et al. 2004, Lakio et al. 2009). Bearing in mind that circulating IgA has a shorter half-life than IgG, the high IgA antibody levels most likely indicate recent or repeated bacteremia (Boillot et al. 2016, Brekke & Sandlie 2003). It has been postulated that IgG, being more constant, displays persistence or recurrence of periodontitis, while IgA antibodies are elevated only during active phase of the disease (Pussinen et al. 2007a). It was previously proposed that chronic periodontitis has dynamic states of exacerbation and remission, and that the increase or the decrease in bacterial burden and inflammatory response may not occur simultaneously but consecutively at different sites of periodontal tissues (Gursoy et al. 2011). Hence, a combination of various parameters might be more accurate in surrogate periodontal diagnostics (Nagarajan et al. 2017).

1.8.2 Autoimmunity

Even though the immune systems main function is to protect us from the environment, several immunopathological processes prevail, causing inappropriate hypersensitivity reactions to exogenous antigens (allergy), loss of tolerance self-antigens (autoimmune diseases), or reactions to foreign grafts (rejections to implants or transplants) (Delves et al. 2011). Many oral species elicit a polyclonal B-cell response, resulting in auto-reactive antibodies directed against collagen and connective tissue proteins and partaking in periodontal tissue destruction (Newman et al. 2002). Apart from local deleterious effects, cross reactive antibodies are likely to influence other tissues also.

Cross activation of auto-reactive lymphocytes due to sequence similarities in host- and pathogen derived peptides is called molecular mimicry. The best documented example of this process concerns human heat shock protein 60 (hHSP60), expressed on macrophages, smooth muscle cells (SMCs) and endothelial cells, and bacterial HSP GroEL, expressed on several oral pathogens (Seymour et al. 2007, Teles & Wang 2011). Antibodies produced against hHSP60 are, for example, consistently linked to

atherogenesis via induction of endothelial dysfunction (Epstein et al. 2009), especially when combined with other cardiovascular risk factors, such as high cholesterol levels (Seymour et al. 2007). Patients with periodontitis have elevated serum levels of *P. gingivalis* - (Tabeta et al. 2000) and *F. nucleatum* specific cross-reactive GroEL antibodies, which might independently induce expression of chemokines and adhesion molecules, and formation of foam cells (Lee et al. 2012).

Other antibody classes displaying cross reactivity between host and bacteria are antiphospholipid antibodies. They are considered atherogenic, and the main antigens include cardiolipin, oxidized low-density lipoprotein (ox-LDL), β2-glycoprotein I and prothrombin. Ox-LDL is found in atherosclerotic plaques and antibodies towards it enhance the accumulation of modified low-density lipoprotein (LDL) into macrophages *in vitro* (Vaarala 2000). Besides different epitopes of ox-LDL antibodies (Buhlin et al. 2015), patients with periodontitis have elevated levels of anti-cardiolipin antibodies, which may at least partly be produced against homologous structures of *A. actinomycetemcomitans*, promoting vascular inflammation (Wang et al. 2008). Mouse models have confirmed cross-reactivity between *A. actinomycetemcomitans* HSP60, *P. gingivalis* gingipain epitopes and ox-LDL (Turunen et al. 2012, Wang et al. 2016). These, along with anti-phosphorylcoline antibodies, all represent potential mechanisms of molecular mimicry, linking periodontal infections with atherogenesis (Schenkein & Loos 2013).

1.9 Atherosclerotic vascular diseases

Atherosclerosis is a highly prevalent chronic slowly progressive disease, characterized by accumulation of lipids and fibrous elements in the large arteries. It is considered an inflammatory disease that might develop acute clinical events by plaque rupture and thrombosis (Lusis 2000). Atherosclerosis is the primary cause of CVDs and stroke and accounts for approximately 50% of all deaths in westernized societies. Atherosclerosis is accounted for 17.3 million deaths per year, making it the leading cause of death worldwide (Lusis 2000, Mendis et al. 2011).

1.9.1 Etiology

Atherosclerosis is a multifactorial disease with a complex etiology. The early “fatty streak” lesions are characterized by subendothelial accumulation of cholesterol-engorged macrophages, called foam cells. These early lesions are commonly found in the aorta already in the first decade of life and due to the progressive nature, coronary arteries and cerebral arteries are usually manifested in the second and third decade, respectively. Atherosclerosis in the coronary arteries is commonly called coronary artery/heart disease (CAD/CHD), and these terms are frequently used interchangeably. Accumulation of lipid rich necrotic debris and SMCs and a fibrotic cap constitute the formation of “fibrous lesions”. Further progression of disease lead to calcification, luminal surface ulceration and hemorrhage of small vessels and total growth of lesion size. Atherosclerotic lesions might obturate blood flow, leading to transient ischemic complications, or alternatively the plaque might rupture or erode, leading to thrombus formation, myocardial infarction or stroke (Lusis 2000).

Several risk factors are associated with atherosclerosis and CAD development. Factors with a genetic component include: elevated levels of LDL/ very low density lipoprotein (VLDL)/lipoproteins/ homocysteine/hemostatic factors, reduced levels of HDL, elevated blood pressure, family history of CAD, diabetes, obesity, metabolic syndrome (MetS), male gender and systemic inflammation. Purely environmental risk factors include high fat diet, smoking, physical inactivity or infectious agents. CHD is commonly formed by a combination of unhealthy environmental factors in genetically susceptible individuals. (Lusis 2000) Inflammation has shown to playing a primordial role in atherogenesis (Ross 1999, Libby 2012). Distant infectious foci are believed to augment a local inflammatory reaction in the atherosclerotic plaque, but also a variety of autoimmune and autoinflammatory diseases are likely to induce a proatherogenic activation of the immune system (Libby et al. 2016).

The role of infectious agents, such as chronic oral infections, have lately been emphasized, while they can cause systemic inflammation and oxidative stress (Rosenfeld & Campbell 2011). Many bacterial species, such as *Chlamydia pneumoniae*, *Helicobacter pylori* and several periodontal pathogens, have been linked to atherogenesis. Hence, a concept of “infectious burden” as a cardiovascular risk factor has emerged, stating that an aggregate burden of atherogenic pathogens might better reflect CVD risk than any single infection (Epstein et al. 2000, Sessa et al. 2014). In this regard, oral microbiological

measures and their systemic immune response have been suggested to better reflect systemic exposure of periodontitis, compared to traditional diagnostic measures (Beck et al. 2005a, Mustapha et al. 2007).

The disseminating systemic effects of oral bacteria are discussed previously (chapters 1.4.3 and 1.5.3). Examples of direct infection induced effects of bacteria on atherogenesis include infection of macrophages, SMCs, and endothelial cells, induced expression of ROS, cytokines (IL-6, IL-1β and TNF-α, etc.), growth factors (basic fibroblast growth factor, tumor growth factor β, etc.) and cellular adhesion molecules (**Figure 5**) (Kebschull et al. 2010). These effects partake in all stages of atherogenesis, plaque rupture and thrombus formation by promotion of endothelial dysfunction, foam cell formation, SMC proliferation and platelet aggregation (Sessa et al. 2014). Endotoxemia is considered an atherosclerotic risk factor by induction of proinflammatory signaling (Stoll et al. 2004). The indirect proatherogenic effects of oral infections include increased systemic inflammation and molecular mimicry, as previously discussed. Cross-reactivity with two human epitopes, ox-LDL and hHSP60, have especially been linked to both oral infections and atherogenesis (Hansson & Libby 2006, Hansson & Hermansson 2011).

1.9.2 Phenotypes, complications and clinical diagnostics

Atherosclerosis is a slowly progressive disease and the vascular lesions are classified by their current histopathological stage. The progressive advanced plaques are responsible for clinical disease and are usually divided into 1) pathologic intimal thickening (with/without erosion), 2) fibrous cap atheroma (with/without erosion, 3) thin-cap fibroatheroma (with/without rupture), 4) calcified nodule and 5) fibrocalcific plaque (Insull 2009). The degree of lesion disruption determines the ensuing clinical state. When only the endothelial surface is disrupted, there is usually no clinical symptoms. In the cases of deeper lesions, a transient and repetitive thrombosis might ensue causing transient vessel occlusion and ischemia, as with patients with unstable angina pectoris. Recurrent thrombosis overlying fissured lesions might lead to a more severe form of vessel occlusion, and manifests clinically as infarction or, in the worst case, sudden death. Deep ulcerated lesions, where the lipid core, tissue factors and collagen are exposed, lead to a persistent thrombotic occlusion and an acute myocardial infarction

(AMI). The thrombogenic potential is generally more dependent on lesion characteristics than size, considering that coronary events are most often associated with 35-65% occlusion by coronary stenosis (Stary et al. 1995).

Stable CAD is usually defined as a state where the patient has stable symptoms and in addition proof of cardiac ischemia or significant coronary artery stenosis. The symptoms may include chest pain (angina pectoris) or shortness of breath (dyspnea). Cardiac ischemia is usually detected by electrocardiogram (ECG), either in rest or stress (Porela et al. 2015). The level of stenosis might be evaluated with computer tomography or coronary angiography imaging, which is nowadays considered the golden standard for accurate determinations (Bassand et al. 2007).

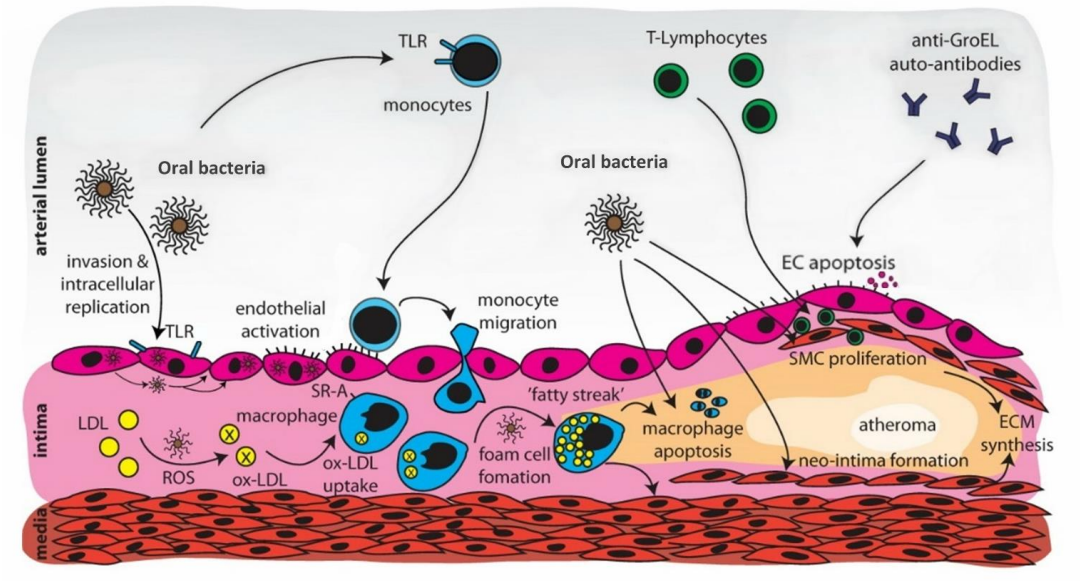

Figure 5. Potential mechanisms linking oral infections and fatty-streak formation/plaque maturation.

Monocytes activated by oral pathogens chemotactically migrate into the subendothelial space, and transform into macrophages and, subsequently, into foam cells after uptake of oxidized LDL. Apoptosis of LDL-laden macrophages results in accumulation of lipids in the subendothelial space. Furthermore, oral bacteria induce smooth-muscle-cell proliferation in the intima and neo-intima formation. Extracellular matrix build-up and extravasation of T-cells consummate the formation of a fibrous cap covering the plaque. Denudation of the fibrous cap and its pro-thrombotic components occurs after endothelial cell apoptosis mediated by oral bacteria, or cross-reactive auto-antibodies (Kebschull et al. 2010). Reprinted and modified with permission from SAGE Publications.

Traditionally, a narrowing of ≥50% of the left main coronary artery or ≥70% in any of the other coronary arteries are considered hallmark features of stable CAD. The total set of diagnostic tests are selected individually, depending on the assessed risk (Montalescot et al. 2013).

Acute coronary syndrome (ACS) is traditionally characterized by an inflammatory atherosclerotic plaque rupture or erosion, although recent studies propose that ACS might also be caused by plaque fissure without inflammation or arterial vasospasm without thrombus. The atherosclerotic plaque activity is known to be modulated by a local imbalance in adaptive immune pathways. (Crea & Libby 2017) ACS is commonly characterized with differing degrees of superimposed thrombosis and distal embolization, resulting in myocardial underperfusion and critical reduction of blood flow. ACS is crudely subdivided into patients with ST-elevation myocardial infarction (STEMI), non-ST elevations myocardial infarction (NSTEMI) or unstable angina. Cell injury and myocardial infarction in ACS patients is commonly determined with elevated blood troponin levels (cTnT/cTnI), although cardiac enzymes, such as creatinine kinase or its isoenzyme MB, are also traditionally measured (Bassand et al. 2007, Vaara et al. 2012).

Stroke is an acute and devastating manifestation of atherosclerosis and hypertension, where cerebral blood flow is hindered leading to cell death. Stroke is defined by the World Health Organization as a "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin" (WHO 1988). In the clinical setting, suspected stroke is always an emergency and requires immediate neurologic evaluation, aiming to start fibrinolytic treatment within 60 minutes (Jauch et al. 2013).

Stroke is generally divided into ischemic or hemorrhagic cerebrovascular disturbances. Ischemic stroke develops mainly due to atherosclerosis in cervical/cerebral arteries or cerebral thromboembolism originated from other parts of the body. Hemorrhagic stroke subforms, such spontaneous intracerebral hemorrhages or cortical amyloid angiopathy, are mainly associated with hypertension and old age (Truelsen et al. 2006). Globally, in 2013 there were almost 25.7 million stroke survivors (71% with ischemic stroke [IS]), 6.5 million deaths from stroke (51% due to IS) and 10.3 million new strokes (67% IS) (Feigin et al. 2017). Approximately 11,500 new stroke cases were

detected in Finland in 2000, and it is believed to be an increasing health issue due to ageing of the population (Sivenius et al. 2009).

1.10 Oral infections and CVDs

The first evidence that oral infections associate with heart diseases was reported by Mattila et al. in 1989. They showed in a cross-sectional study, that a total dental index, based on caries, periodontitis, periapical lesions and pericoronitis, significantly associated with myocardial infarction (Mattila et al. 1989). This finding inspired a vast line of research with a clear emphasis on associations between marginal periodontitis and CVDs. To date, the association between periodontitis and CVDs is well established, independent of confounders and consistent through different populations globally (Kebschull et al. 2010, Lockhart et al. 2012). In 2012, the American Heart Association stated in a scientific report, that periodontitis has a confounder independent association with atherosclerotic vascular disease (Lockhart et al. 2012). Periodontitis has also shown to associate with coronary heart disease (Mattila et al. 1993, Bahekar et al. 2007, Humphrey et al. 2008), myocardial infarction (Mattila et al. 1989, Rydén et al. 2016), carotid atherosclerosis (Beck et al. 2001, Zeng et al. 2016), ischemic stroke (Grau et al. 1997, Janket et al. 2003) and endothelial dysfunction (Amar et al. 2003, Higashi et al. 2009), all representing clinical manifestations of atherosclerosis.

All tough a plethora of epidemiological-, intervention-, *in vitro*- and animal studies support an association between oral infectious diseases and CVD, and have proposed several plausible mechanistic links, statements of true causality are still unwarranted (Lockhart et al. 2012, Tonetti et al. 2013, Schenkein & Loos 2013). Per Hill's criteria of causality in medicine, true statement of causality requires that nine different criteria are met (Hill 1965). Some of these are not fully justified in this context, for example, the deleterious effects of oral inflammatory diseases are not specific for CVDs, and temporality is hard to prove between two slowly progressive inflammatory diseases due to the long observation times required (Kholy et al. 2015). Even though we already have some evidence that periodontal treatment reduces serum levels of inflammatory mediators and improves endothelial function (Tonetti et al. 2007, Teeuw et al. 2014), these are merely arbitrary markers for subclinical CVD. Well conducted randomized controlled trials in humans, the golden standard for assessing

temporality, are not possible to conduct in this regard due to ethical reasons (Belstrøm et al. 2012). Nevertheless, it is vital that vivid research continues to explore the associations between oral infections and cardiometabolic disorders, considering the high prevalence of these diseases, which have a major impact from a population health perspective (Cullinan & Seymour 2013).

AP might contribute to atherogenesis through similar pathways as with marginal periodontitis, due to similarities in inflammatory and microbial profiles as previously discussed (chapter 1.5.3). This line of research was initiated by Frisk et al. in 2003 (Frisk et al. 2003), and has lately increased awareness. Even tough results have been somewhat inconsistent, most reports from cross-sectional and longitudinal analyses show an evident positive association between AP and CHD (Caplan et al. 2006, Joshipura et al. 2006, Caplan et al. 2009, Pasqualini et al. 2012, Petersen et al. 2014, Costa et al. 2014). Recently, a few systematic reviews have postulated that this association is conceivable, with a low to moderate grade of evidence (Berlin-Broner et al. 2016), and further well conducted longitudinal studies are warranted for confirmation of the hypothesis (Khalighinejad et al. 2016).

1.10.1 Common risk factors and genetics

Oral infectious diseases and CVDs share several risk factors. These should be considered as confounding variables in statistical analyses, as an observed association might depend on similar patient characteristics. Imbalances in risk factors for the study outcome in different exposure groups result in statistical confounding. Effect modification, or interaction, occurs when the effect of the risk factor on an outcome differs depending on the value of another variable. Assessing potential confounding or effect modification is essential in epidemiologic surveys, for example by means of restriction of study population or adjustment of statistical models with covariates (Hyman 2006).

Destructive periodontitis is a consequence of the interaction of genetic, environmental, host and microbial factors. Documented risk factors for marginal periodontitis includes the local microbiota, increase in age, male gender, low socioeconomic status (SES), smoking, diabetes, stress (low grade of evidence) and genetics (Kinane 2001). The total risk is indirectly modified by lifestyle factors. The risk for AP correlates with increasing age (Hussein et al. 2016), but is mostly affected by local factors, such

as caries and its associated microbiota, RCFs (especially if poor quality), tooth crown restorations and ABL (Kirkevang et al. 2007, Ingle et al. 2008, Kirkevang et al. 2017).

The best documented shared risk factors for marginal periodontitis and atherosclerosis are increasing age, male sex, smoking, race/ethnicity, education and SES, diabetes, alcohol abuse, obesity/body fat content, and possibly stress (Peacock & Carson 1995, Hujoel et al. 2000). Other factors that ought to be considered are lifestyle factors, such as health behavior and diet (Lockhart et al. 2012), metabolic inflammation and genetics (Janket et al. 2015). In addition, a heterogenic plethora of adjusted variables have been used in this context, such as lipoprotein composition, blood pressure, medications, dental visits, number of teeth present and family history of systemic disease. As a very minimum, age and gender should be considered (Dietrich et al. 2013). Lastly, one should bear in mind the everlasting possibility of "unknown confounders", which are yet to be discovered and cannot be accounted for (Kebschull et al. 2010).

Both periodontitis (Mucci et al. 2005) and CVDs (Kessler et al. 2016) have a high degree of heritable components. Attempts in identifying periodontitis associated loci in genome-wide association studies (GWAS) have failed to reach GWAS significance, but proposed several candidate genes (Divaris et al. 2013). Chronic periodontitis has been shown to associate with polymorphism in the genes IL1, IL6, IL10, VDR, CD14, TLR4 and MMP1 and the heritability of periodontitis might be as much as 50% (Laine et al. 2012). Variations in the MHC region, essential for innate and adaptive immunity, might also be important in susceptibility for periodontitis (Kallio et al. 2014). The pathogenesis of AP is probably less affected by genetics, although there is some evidence stating that polymorphism and biological modifiers induce a more vigorous immunoinflammatory response which affects the periapical disease phenotype (Aminoshariae & Kulild 2015).

Genetic markers and variants associated with periodontitis and their overlap with CAD was recently reviewed by Aarabi et al. (2017) (Aarabi et al. 2017). To date, GWAS have identified more than 50 genes associating with premature CAD. Four loci are associated with aggressive and/or chronic periodontitis: VAMP3/CAMTA1, PLG, ANRIL/CDKN2A/CDKN2B and GLT6DI, and several candidate genes require further validation. Most of the associated genetic risk variants are located in introns, representing regulatory elements and intergenic regions, and their molecular mechanisms in

periodontal etiology are yet to be elucidated. Three out of four periodontitis associated gene variants, namely PLG, ANRIL, and VAMP3/CAMTA1, are also associated with CAD / AMI. This is a strong indication for that both diseases may be caused concurrently due to activation or downregulation of common pathways, regulating coagulation, inflammation, and immunity, all of which are mechanism involved both in periodontitis and atherosclerosis (Aarabi et al. 2017). Even tough shared genetic factors between CVD and periodontitis have been found, the association between periodontitis and CVDs is little affected after adjustments with genetic factors (Mucci et al. 2009).

1.10.2 Suggested biological mechanisms

Several mechanistic links between periodontitis and CVDs have been proposed and the main hypotheses were reviewed by Schenkein & Loos in 2013 (Schenkein & Loos 2013). The chronic oral infections cause bacteremia and periodontal pathogens have been frequently found in atheromatous plaques, where they are suspected to invade endothelial cells, provoke inflammation, induce procoagulant effects and endothelial dysfunction, thereby promoting atherogenesis as so called "direct effects" (Cullinan & Seymour 2013). Several oral bacteria are capable of invading arterial endothelial cells (Teles & Wang 2011). The best documented species is in this regard is *P. gingivalis*, which causes upregulation of genes coding many pro-inflammatory cytokines, adhesion molecules and E-selectin (Chou et al. 2005). Noteworthily, not only periodontal pathogens have been detected in atheromas, but also other oral residents, such as etiological species to dental caries (Kozarov et al. 2006). However, the exact role these bacteria have in atherogenesis is yet to be elucidated.

Oral infections increase systemic levels of inflammatory markers and LPS, either locally produced or via bacteremia, as previously discussed (chapters 1.4.3, 1.5.3, 1.7). These are known to play a significant part in the etiology of atherosclerosis (Stoll et al. 2004, Libby 2012), and represent so called "indirect effects" between periodontitis and CVDs (Teles & Wang 2011, Hansson et al. 2015). Molecular mimicry by cross reactive circulating antibodies, enhances endothelial inflammation, promotes uptake of lipids into macrophages, and blocks antiatherogenic effects of protective molecules (Pussinen et al. 2003, Schenkein & Loos 2013). Serum anti-hHSP antibodies have been shown to correlate with the presence and extent of CVDs (Zhu et al. 2001). Furthermore, periodontitis

has been proposed to affect the lipoprotein profile into a more proatherogenic direction (Pussinen & Mattila 2004, Kallio et al. 2013). Patients with periodontitis have repeatedly shown to have elevated triglyceride levels and decreased HDL levels, indicating proatherogenic dyslipidemia (Lamster & Pagan 2017).

It is likely that a total pathogen burden poses a greater risk for atherogenesis than any single pathogen (Zhu et al. 2000). This notion is supported by the findings that an aggregate subgingival burden of oral pathogens associates with increased carotid intima-media thickness (Desvarieux et al. 2005) and angiographically determined CHD (Spahr et al. 2006). Circulating antibodies against periodontal pathogens indicate an increased risk for CHD (Pussinen et al. 2003) and it has been proposed that these antibodies better reflect the systemic exposure to periodontal infections, compared to subgingival measurements alone (Beck et al. 2005a, Mustapha et al. 2007). However, the relationships of the aggregate periodontal pathogen burden, their immune responses and CAD have not been properly elucidated.

1.10.3 Missing teeth and CVDs

Advanced tooth loss might be considered a marker for past, present or treated oral inflammatory diseases (Desvarieux et al. 2003, Holmlund & Lind 2012). Edentulousness represents an exceptional end stage, eliminating all clinical evidence of ongoing inflammation, even though the systemic damage may be irreversible and evident (Hujoel et al. 2001a). Missing teeth have been frequently associated with atherosclerotic complications, such as CHD (Pasqualini et al. 2012), coronary atherosclerotic burden (Gomes et al. 2012b), myocardial infarction (Holmlund et al. 2017a), arterial stiffness (Asai et al. 2015), carotid calcifications (Desvarieux et al. 2003, Holmlund & Lind 2012), self-reported CVD (Wiener & Sambamoorthi 2014), stroke (Vedin et al. 2015) and total CVD mortality (Ragnarsson et al. 2004, Cabrera et al. 2005, Polzer et al. 2012, Watt et al. 2012, Schwahn et al. 2013). Of note, most of past studies on association between missing teeth and CVDs are based on surrogate variables or CVD-mortality as the outcome, and more research is needed on actual CVD incidence.

1.11 Periodontitis and diabetes

Diabetes is a group of metabolic disorders characterized by hyperglycemia. Periodontitis and diabetes have been suggested to have a bi-directional association, where periodontitis increases the risk for diabetes and vice versa. Diabetes leads to a hyperinflammatory response to the periodontal microbiota and impairs resolution of inflammation and tissue repair, probably due to impaired neutrophil function and actions of advanced glycation end products in the periodontal tissue (Lalla & Papapanou 2011, Nagpal et al. 2015). Accumulating evidence shows that inflammation is a key player in the pathogenesis of diabetes (Shoelson et al. 2006), and the systemic inflammation caused by oral infections might therefore impede glycemic control and promote insulin resistance (Lalla & Papapanou 2011). Marginal periodontitis has been suggested to be an independent predictor for incident diabetes (Demmer et al. 2008, Winning et al. 2017), and periodontal therapy might reduce systemic inflammatory markers in diabetic subjects (Artese et al. 2015). However, there is currently little evidence on how periodontitis or number of missing teeth contributes to incident diabetes. Diabetic patients have an increased risk for periodontal disease (Chavarry et al. 2009), which is sometimes called “the sixth complication” of diabetes (Saini et al. 2011). Missing teeth may also associate with diabetes (Demmer et al. 2008), although literature on that topic is scarce to date.

2 Aims of the study

The general aim of this thesis was to investigate associations between oral infectious diseases, oral bacteria, along with their respective adaptive immune response, endotoxemia and CVD outcomes. The general hypothesis was that marginal and apical periodontitis increase the risk for CVD events due to bacterial mediators and that missing teeth represents a useful surrogate marker for a history of oral diseases.

The specific aims were:

I. To explore salivary *A. actinomycetemcomitans* and *P. gingivalis* levels and their corresponding serum antibody levels as a diagnostic tool for periodontitis.

II. To study how serum antibodies to selected periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, *P. endodontalis*, *T. forsythia*, *C. rectus*, *P. intermedia*) and the bacterial levels in subgingival samples associate with each other, chronic periodontitis and angiographically verified CAD.

III. To study the association between subgingival bacterial burden, salivary and serum LPS activity and how periodontitis and CAD interrelates with them.

IV. To investigate the associations between AP, CAD, subgingival *P. endodontalis* and its corresponding serum antibodies.

V. To analyze associations and prediction value between missing teeth and incident CVD events, incident diabetes and death.

3 Study subjects and methods

3.1 Study subjects

3.1.1 The Health 2000 survey (I)

The Health 2000 survey was an extensive Finnish population-based survey conducted by the National Institute for Health and Welfare of Finland (formerly National Public Health Institute). A nationally representative random two-stage stratified cluster sample of N=8,028 adults (≥ 30 years of age) was collected during 2000-2001 for evaluation of changes in the health status, functional capacity and welfare of the population. Information of study subjects was gathered by structured health interviews and collection of blood samples in 80 different Finnish health center regions, representing all five university hospital regions (Suominen-Taipale et al. 2008). Thorough clinical oral examinations, along with panoramic radiographs, were conducted for 5,245 dentate subjects. The gingival sulcus of all teeth, except for third molars and residual teeth, were probed from four points: 1) facio-distally; 2) facially; 3) oro-mesially; and 4) orally. The deepest measured PPD for each tooth was recorded. Saliva samples were collected for microbiological measurements from 1,294 subjects residing in the southern district premises (Könönen et al. 2007). Serum IgA and IgG levels to *A. actinomycetemcomitans* and *P. gingivalis* were determined by multi-serotype enzyme-linked immunosorbent assay (ELISA) as previously reported (Pussinen et al. 2011).

Study I was based on a subsample of 230 participants (106 males and 124 females; mean age – SD: 48.7 – 5.4 years), with oral examinations and saliva collections. The general inclusion criteria for this subsample were: 1) aged 40 to 60 years 2) having at least 20 teeth intact 3) residing in either of three predefined periodontal subcategories: Periodontally healthy (no deepened periodontal pockets, n=81), localized periodontitis (two or seven teeth with PPD ≥ 4mm, n=65) and generalized chronic periodontitis (≥ 14 teeth with PPD ≥ 4mm, n=84). The study groups were selected to produce a sample representing different phenotypes of chronic marginal periodontitis. The levels of salivary *A. actinomycetemcomitans* and *P. gingivalis* were determined for this subsample with quantitative single copy gene-based real time polymerase chain reaction (qPCR) (Hyvärinen et al. 2009). All protocols for

the Health 2000 survey were carried out according to the Declaration of Helsinki, approved by the ethic committees of the Helsinki University Central Hospital and the National Public Health Institute and the patients signed an informed consent.

3.1.2 The Parogene study (II, III, IV)

The Corogene study was a large prospective cohort of N=5,297 Finnish adults who underwent coronary angiography for any reason at the Helsinki University Central Hospital between June 2006 and March 2008. Patients with non-Finnish origin, previous heart transplantation, low hemoglobin or previous blood transfusion during the same hospitalization were excluded. In Finland, coronary angiography is performed for practically all patients assigned for invasive heart examinations. The main purpose of the Corogene study was to follow contemporary trends in coronary heart disease, heart disease related risk factors, genetics and epigenetics (Vaara et al. 2012).

A random sex-stratified subsample of approximately 10% of the Corogene population was assigned for the Parogene cohort (N=508). The purpose of the Parogene study was to investigate associations between CAD and oral health. Extensive clinical oral health examinations, including PPD measurements from six surfaces of each tooth, were performed by two calibrated periodontal specialists approximately 6 to 20 weeks after the coronary angiography (mean ± SD: 113 ± 30 d; range, 37 to 224 d) (Buhlin et al. 2011).

Subgingival bacterial samples were collected and pooled from all dentate subjects (n=473) from the most diseased site of each dental quadrant during the oral examinations (Mäntylä et al. 2013). Subgingival bacterial levels for 70 species (75 strains) were measured with checkerboard DNA-DNA hybridization (Socransky et al. 2004, Pradhan-Palikhe et al. 2013) and a full list is displayed in Study III (Suppl. table I). Saliva samples were collected as described earlier (Hyvärinen et al. 2012). All dentate and edentulous (n = 32) subjects underwent panoramic tomographies analyzed at the Institute of Dentistry, University of Helsinki. The number of teeth with periapically widened spaces, apical rarefactions or RCF and ABL were recorded by a radiologist specialized in oral and maxillofacial

radiology. A widened periapical space indicates a milder form of AP (PAI=3) and wider lesion (PAI=4-5) represent more advanced phenotypes of AP, and were called apical rarefactions (Ørstavik et al. 1986).

In studies II & III we designed a periodontal diagnosis for each subject: "healthy" (no ABL and BOP<25%, n=46), "gingivitis" (no ABL and BOP≥25%, n=65), "history of periodontitis" (mild-severe ABL and BOP<25%, n=92) and "active periodontitis" (mild-severe ABL, BOP≥25%, n=269). In study IV, we designed a 3-step score for each subject according to AP as follows: score I, no AP (n=210, including 32 edentulous subjects); score II, ≥1 widened periapical space and/or 1 apical rarefaction (n=222); score III, ≥2 apical rarefactions (n=76). The groups were selected to reflect a continuum of the patients' infectious endodontic status. In study IV, mild-severe ABL (vs. no ABL) was used as a proxy for marginal periodontitis, as previously reported for the same population (Hyvärinen et al. 2012, Pradhan-Palikhe et al. 2013).

The population was grouped according to cardiologic diagnosis and results from the coronary angiography; no significant CAD (<50% stenosis of the coronary arteries, n=123), stable CAD (≥50% stenosis in at least one coronary artery, n=184), ACS (having an episode of chest pain, typical ECG changes, elevated levels of cardiac biomarkers and ≥50% stenosis in at least one coronary artery; n=169), and ACS-like but no significant CAD (having chest pain w/wo ECG changes but <50% stenosis of the coronary arteries, n=32) (Buhlin et al. 2011). This grouping was used in studies III and IV. In study II, patients with "no significant CAD" and "ACS-like but no significant CAD" were grouped together, all representing patients with <50% stenosis in the coronary arteries.

Blood samples were drawn at the time of the angiography and stored at −80 C°. Salivary and serum LPS activity was analyzed with Limulus amebosyte lysate (LAL) assay coupled with a chromogenic substrate (Hyvärinen et al. 2012). Serum levels of Ig A and G (IgA/IgG) against whole cell antigen of *A. actinomycetemcomitans, P. gingivalis, F. nucleatum, P. endodontalis, T. forsythia, C. rectus* and *P. intermedia* were determined with ELISA (Pussinen et al. 2002). The Helsinki University Central Hospital Laboratory analyzed the high sensitivity C-reactive protein.
Additional data, such as information on weight, height and smoking, was collected by questionnaires and hospital records. Diagnosis for hypertension, dyslipidemia and diabetes was based on respective current medications. The Parogene study complies with the principles of the Declaration of Helsinki

and informed consent was obtained from all study subjects. The study design was approved by the Helsinki University Central Hospital ethics committee.

3.1.3 The FINRISK 1997 study (V)

The national FINRISK 1997 study is a large population-based cohort (N=8,446) on non-communicable diseases and their risk factors in subjects 25 to 74 years of age (Vartiainen et al. 2010, Borodulin et al. 2015). The risk factor survey has been conducted by the National Institute of Health and Welfare with a 5-year interval since 1972, using independent, random and representative population samples from different parts of Finland. Formerly it was known as the North Karelia Project, and the FINRISK study has yielded over 1200 scientific publications to date. The survey methods are in concordance with the World Health Organization's (1988) MONICA protocol (WHO 1988). FINRISK 1997 includes data collection from all five major Finnish districts by a comprehensive questionnaire and participation in a clinical examination. In the baseline examinations, the number of teeth present was counted by a trained nurse, equipped with a flashlight and a spatula. Also, venous blood samples were drawn and weight, height, and blood pressure measurements were conducted. Information on nutritional intake was collected from a 24-h dietary recall, and available for a subset of N=2,452, constituting the FINDIET97 survey (Kallio et al. 2015).

In study V, information on all used covariates, excluding macronutrient intake, was available for N=7,862 individuals. The cases with prevalent CVDs or diabetes at baseline were identified via 1) the questionnaire as a doctor-diagnosed disease; 2) record linkage with the disease-associated drug reimbursement records from the Social Insurance Institution of Finland, including purchased medications and entitled reimbursements; and 3) record linkage with the National Hospital Discharge register for hospitalizations. In our study, fulfilling any of these identified the subject as having existing CVD or diabetes. Study V utilized > 13 years (median 13.8 years) of follow up data for endpoints; CVD and diabetes incidence, and all-cause mortality. Follow up information was gathered using record linkage with the 1) drug reimbursement records; 2) National Hospital Discharge register; and 3) National Causes-of-Death Register (Pajunen et al. 2004). The studied end points included

diabetes (n=557), all cause death (n=891) and CVD-events (n=692), which are further divided into CHD-events (n=482), AMI (n=253) and stroke (n=268).

The study was approved by the ethics committee of the National Public Health Institute and conducted according to the Helsinki Declaration. All subjects gave an informed consent.

3.2 Methods

A summary of the methods used in the studies I-V is presented in **Table 1**. The main methods are explained in detail below.

3.2.1 qPCR (I)

Whole saliva samples were collected by paraffin-stimulated expectoration before the oral examinations, and divided into Eppendorf tubes. Thereafter, the samples were frozen in carbonic acid ice for transportation and stored at -70°C until used. Bacterial quantification for study I was performed partially earlier (Hyvärinen et al. 2009), and partially during this thesis project for patients with localized periodontitis (n=65).

The qPCR protocol used in study I is previously reported in detail (Hyvärinen et al. 2009). Total bacterial DNA was extracted from the saliva samples using the ZR Fungal / Bacterial DNA kit™ (Zymo Research) according to the manufacturer's instructions. The purity of the reference bacterial cultures was controlled with microscopic evaluation and Gram staining, whereafter the colonies were suspended in 200 µl 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, 1 mM ethylenediaminetetraacetic acid (EDTA) buffer and DNA was isolated. The saliva samples were centrifuged (9300xg, 3 min), the supernatants were removed and the pellets were suspended in 200 µl 10 mM Tris, pH 8, 1 mM EDTA buffer. The DNA were isolated in 70 µl elution buffer. The DNA concentrations were measured with ND-100 (NanoDrop Technologies).

Table 1. Summary of methods used in the studies

Method	Publication
Clinical oral examinations	I, II, III, IV
Radiographic oral examinations	I, II, III, IV
Bacterial cultures	I, II
Quantitative polymerase chain reaction (qPCR)	I
Enzyme-linked immunosorbent assay (ELISA)	I, II, IV
Checkerboard DNA-DNA hybridization	II, III, IV
Limulus amebocyte lysate assay (LAL)	III, IV
Record linkage with national registers	V
Statistical analyses, main tests	
χ^2 test	I, II, III, IV, V
Kruskal-Wallis test	I, III, IV
Fisher exact test	I
Mann-Whitney U test	II, III, V
1-way analysis of variance (ANOVA)	I, III, V
2-way ANOVA	IV
Pearson's correlation	III
Linear regression	I, II, III
Logistic regression (binomial / multinomial)	I, II, III, IV, V
Receiver operating characteristic (ROC)	I
Normalization (log-transformation / Z-score)	I, II, III, V
Factorial ANOVA	II
Jonckheere-Terpstra test	IV
Cox regression	V
Net reclassification improvement (NRI)	V

Primers (produced by Thermo Scientific) and probes (TaqMan, produced by Thermo Scientific and Oligomer) were designed with EPRIMER3-software (**Table 2**). The quantitative single copy gene-based real-time PCR was performed with Mx3005P Real-Time QPCR System (Stratagene). DNA concentrations for *A. actinomycetemcomitans* and *P. gingivalis* were determined from all samples as duplicates. The primer/probe concentrations were 100 nM/200 nM for *A. actinomycetemcomitans* and 300 nM/200 nM for *P. gingivalis.* The thermocycling program was 10 min at 95°C (initial denaturation), 40 cycles of 30 s at 95°C, and 1 min at 60°C. The fluorescence increase was monitored during PCR amplification and all data, including the reaction efficiency determinations by standard curve slopes, were analyzed using the software of Mx3005P Real-Time QPCR System (Stratagene).

Table 2. Primer and probe sequences

Pathogen	Primers 5'→ 3'	Probe 5'→ 3'	Target gene
A. actinomycetemcomitans	F: GCGAACGTTACGCGTTTTAC	AATTGCCCGCACCGAAACCCAAC	*waaA*
	R: GGCAAATAAACGTGGGTGAC		
P. gingivalis	F: TGGTTTCATGCAGCTTCTTT	CGTACCTCATATCCCGAGGGGCTG	*waaA*
	R: TCGGCACCTTCGTAATTCTT		

3.2.2 ELISA (I, II, IV)

Serum levels of IgA and IgG against all studied bacteria were determined with ELISA. The used protocol was similar for study I (Health 2000 survey) and studies II and IV (the Parogene survey) and originally reported (Pussinen et al. 2002). *A. actinomycetemcomitans* and *P. gingivalis* IgA/IgG determinations were available for study I from the research group's earlier study (Pussinen et al. 2011), as well as *A. actinomycetemcomitans* IgA/IgG for studies II and IV (Hyvärinen et al. 2012). The remaining analyses, i.e. serum IgA/IgG determinations for *P. gingivalis* (II), *P. endodontalis* (II, IV), *P. intermedia* (II), *T. forsythia* (II), *C. rectus* (II) and *F. nucleatum* (II), for the Parogene population were performed and first reported during this thesis project. The main features for this indirect ELISA are explained below. The ELISA assays of *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum* were

designed to detect antibody levels against different bacterial strains, other antibody levels were measured by using one strain-derived antigens. The used strains are reported in **Table 3**.

Table 3. Pooled bacterial strains, dilutions and inter assay CV used in the ELISA assays

	Strain	Serum dilution IgA	Serum dilution IgG	Inter-assay CV %[†]
A. actinomycetemcomitans	29523*, a 43718*, b 33384*, c IDH781, d IDH1705, e C59A**	1:100/1:200	1:1500/1:3000	4.7
P. gingivalis	33277*, a W50, b OMGS434, c	1:100/1:200	1:100/1:200	6.9
P. endodontalis	35406*	1:100/1:200	1:100/1:200	7.9
P. intermedia	25611*	1:100/1:200	1:1500/1:3000	7.8
T. forsythia	43037*	1:100/1:200	1:1500/1:3000	8.3
C. rectus	33238*			
F. nucleatum	25586* 33693* 10953* 49256*	1:1500/1:3000	1:1500/1:3000	7.3

* ATCC; letters a-e after the strain refer to serotype; ** non-serotypeable strain; [†] Parogene population; CV, coefficient of variation; ELISA, Enzyme-linked immunosorbent assay.

All bacterial strains were first cultivated on Brucella agar plates (containing 5% horse blood, hemin [5 µg/ml], vitamin K1 [100 mg/ml], and Brucella agar) in their respective optimal growing environments, for two to six days. Regarding *A. actinomycetemcomitans,* the colonies were incubated on Brucella agar plates in an atmosphere of 5% CO2 at 37°C and further incubated in Todd-Hewitt broth (3% TH, 1% yeast extract). The remaining species were cultivated in anaerobic conditions, and all bacterial colonies were isolated by centrifugation. The purity of the cultures was controlled by microscoping the colony morphology and standard Gram staining. The cultures were transferred to a fixing solution,

phosphate buffered saline (PBS, 10 mM phosphate [pH 7.4], 150 mM NaCl) containing 0.5% formalin, and incubated overnight at +4°C. The acquired cultures were washed three times with PBS, followed by centrifugation at 5000 rpm for 10-15 minutes and extraction of the supernatant in between. The washed antigens were preserved in PBS at +4°C.

The bacterial suspensions were diluted in an antigen buffer (0.1 M carbonate, pH 9.6) and the densities were adjusted to give an absorbance of 0.15 at 580 nm (Ultrospec 2100 pro™, Biochrom™). For bacterial species with several strains included, equal volumes of the each were mixed and used to coat microtiter plates (Cliniplate Uni; Labsystems, Helsinki, Finland). For the plate coating, 100 µl of the antigen suspension was added to each well and incubated for 4 hours at room temperature (RT), and overnight at +4°C. The plates were washed three times with a wash buffer (0.05% Tween 20 in PBS), whereafter 100 µl post-coat buffer (5% bovine serum albumin in PBS) was added to block unspecific binding. The plates were incubated at RT for 30 minutes, emptied, covered, and stored at -70°C until used.

Serum samples were diluted in an antibody buffer solution (PBS, 0.05% BSA, 0.05% Tween 20) and analyzed as duplicates in 1:2 dilutions as depicted on **Table 3**. A set of blanks and control sera, identical to all plates, were included. For antibody-antigen reactions, 100 µl of each serum sample was added to the wells and incubated at RT for 2 hours, whereafter the plates were washed three times with the wash buffer (PBS, 0.05% Tween 20). Horseradish peroxidase (HRP)-coupled goat anti-human IgA or IgG (Sigma) was added (100 µl) and incubated for in RT for 1 hour, adhering to any antibodies bound to the antigen. The plates were again washed 3 times with the wash buffer. A chromogenic substrate solution (OPD, Sigma, P9187) was prepared according to the manufacturer's instructions, added to the wells (100 µl) and incubated for at RT in dark for 45 minutes. The color development was terminated with a stop buffer (3 N HCl). The absorbance values were immediately detected with Victor™ X4 (PerkinElmer) at 492nm. The observed values are assumed to correspond with the initial serum antibody level against the detectable antigen.

The output for the absorbance values were manually inspected, and incoherent samples, for example those with a large variation between duplicates, were repeated. Thereafter, the mean value of blank samples was subtracted. Each serum sample produced a mean value of duplicates and 1:2 dilutions.

Every bacterial species, IgA and IgG separately, acquired a coefficient based on the mean of reference serum samples across all plates. This coefficient was used to normalize the results, yielding comparable absorbance values across all assays. The inter-assay coefficients of variation (CV %) for the Parogene population are visible on **Table 3**.

4 Results and Discussion

4.1 Periodontal diagnostics with oral pathogens and serum antibodies (I, II)

A variety of bacteria have been postulated to be etiologically linked to marginal periodontitis (Socransky et al. 1998, Perez-Chaparro et al. 2014). The most notorious species are without doubt *A. actinomycetemcomitans* and *P. gingivalis*, which are frequently detected subgingivally in higher levels in periodontitis patients compared to periodontally healthy controls (Slots 1999). Saliva is an appealing oral fluid in periodontal diagnostics, especially in population based studies, as it is an easily collectible specimen and less time-consuming as compared to subgingival samples. For example, salivary *A. actinomycetemcomitans* has proved to reflect appropriately with subgingival bacterial levels (Cortelli et al. 2005, Hyvärinen et al. 2009). Whole saliva samples might be even more accurate in detection of pathogen presence, compared to pooled subgingival samples (Umeda et al. 1998). Paraffin-stimulated saliva collection is a commonly used method, which does not significantly alter the measured protein levels (Mohamed et al. 2012) and represents fairly well but are naturally lower than the bacterial counts detected in dental plaque (Motisuki et al. 2005). As new sophisticated techniques develop clinical biomarker diagnostics, saliva might indeed be the media of choice in the future (Zhang et al. 2009). Salivary quantities of *A. actinomycetemcomitans* and *P. gingivalis* were determined with qPCR in study I.

Study I was based on a subset of the Health 2000 population. Demographic variables characterizing chronic generalized periodontitis (CGP) were male gender (60% vs. 34% of periodontally healthy controls), lower SES (71% with basic or secondary level of education vs. 31% of controls), and smoking (80% current smokers vs. 84% of controls), all of which are typical risk factors for periodontitis (Kinane

2001). Patients with CGP were more frequently pathogen carriers (43% *A. actinomycetemcomitans* carriers vs. 26% of controls, 80% *P. gingivalis* carriers vs. 56% of controls) and had higher levels of both studied bacteria ($p<0.001$ in Kruskal-Wallis test). Of note, the vast majority of past studies have studied associations between subgingival samples and CGP, whereas accurate quantitative salivary levels are less studied in this regard. In a population based cross-sectional study including 1,198 Finnish adults, it was concluded that salivary levels of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, and *T. denticola* correlated weakly but significantly with periodontitis, and that the presence of several pathogenic species might have a better predictive value than any single pathogen (Könönen et al. 2007, Paju et al. 2009).

We have compared two bacterial quantification methods in periodontal diagnostics in a preliminary study, based on the Parogene population (Pietiäinen et al. 2017). The amount of *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* were detected both from saliva by qPCR and from subgingival samples by checkerboard DNA-DNA-hybridization. C-statistics, adjusted with age, gender, smoking, and number of teeth, was used to measure the performance of any of the continuous bacterial variables in detection of periodontitis (defined here as ABL and PPD ≥ 4mm at least in four sites, n=131). The highest prediction values were obtained when salivary pathogens (Area under the curve [AUC] 0.738, $p<0.001$) or subgingival pathogens (AUC 0.803, $p<0.001$) were combined with the covariates, suggesting that both saliva and subgingival samples are effective in distinguishing periodontitis, although subgingival bacterial samples provided a somewhat better diagnostic value (Pietiäinen et al. 2017).

Serum antibodies towards periodontal bacteria, especially *A. actinomycetemcomitans* and *P. gingivalis*, have been earlier noted to correlate with the extent of periodontitis with a moderate accuracy, although further studies are warranted to verify the results (Papapanou et al. 2000, Pussinen et al. 2002, Dye et al. 2009, Vlachojannis et al. 2010). A few studies have criticized the proposed diagnostic values of mere serum antibodies, while they are not effective in discriminating principal forms of periodontitis (Hwang et al. 2014), might better reflect an individual's ability to mount immune responses (Dahlen et al. 2016), and better reflect pathogen carriage than clinical disease (Pussinen et al. 2011). In study I, only *P. gingivalis* specific serum IgA/IgG response associated with more progressed periodontal disease.

ROC analyses were conducted for determinations of diagnostic specificity and sensitivity of salivary *A. actinomycetemcomitans* and *P. gingivalis* carriage and corresponding serum IgA/IgG levels in CGP detection, compared to the rest of the population. Results from the crude models are displayed on **Table 4**. Of the studied variables, only salivary *P. gingivalis* levels had a good diagnostic value for CGP detection, although *P. gingivalis* specific antibodies yielded statistically significant results also. This finding is in line with the results from a study by Kudo et al. (2012) (Kudo et al. 2012), where the authors concluded that merely *P. gingivalis* serum IgG titers are suitable for screening of chronic periodontitis.

Dye et al. conducted a study, comprising over 5,700 dentate adults (NHANES III cohort), to evaluate the diagnostic capability of serum IgG levels for any of 19 different periodontal bacteria in detection of periodontitis (Dye et al. 2009). Of all the studied species, *P. gingivalis* antibodies had the strongest positive correlation with a moderate diagnostic accuracy across three different criteria for periodontitis. On the other hand, they concluded that this association was influenced by demographic, behavioral and health related characteristics, and these factors ought to be accounted for in epidemiologic surveys. Combining different host and/or pathogen associated parameters might be the most justified strategy to find a diagnostic test for periodontitis (Pussinen et al. 2011), while periodontitis has been postulated to exhibit dynamic states of exacerbation and remission, with site and time dependent fluctuations in pathogen burden, inflammatory response and tissue destruction (Gursoy et al. 2011). Therefore, a combination of pathogen levels and serum antibodies were hypothesized to improve the diagnostic model in study I.

Table 4. Predictive capability of salivary pathogen levels and serum antibodies for CGP

Pathogen		AUC	95% CI	p-value
A. actinomycetemcomitans	Saliva levels (GE/mL)	0.63	0.56-0.71	**0.001**
	Serum IgA levels (EU)	0.56	0.49-0.64	0.113
	Serum IgG levels (EU)	0.54	0.46-0.62	0.306
P. gingivalis	Saliva levels (GE/mL)	0.79	0.72-0.85	**<0.001**
	Serum IgA levels (EU)	0.65	0.58-0.72	**<0.001**
	Serum IgG levels (EU)	0.63	0.55-0.71	**0.001**

Receiver operating characteristic (ROC) with CGP compared to the rest of the population. CGP, chronic generalized periodontitis (≥ 14 teeth with PPD ≥ 4mm) n=84; AUC, Area under the curve; Ig, immunoglobulin; EU, ELISA units. Bold indicates statistical significance (p< 0.05).

Binary logistic regression was used for c-statistic, which was complemented with typical periodontitis related risk factors. Again, GCP was used as the dependent variable, and the results are displayed on **Table 5**. We discovered that combining information on *P. gingivalis* salivary levels with serum antibodies was a good indicator for CGP detection (AUC 0.74, 95% CI 0.66–0.81, p<0.001), and the diagnostic value was excellent when information of age, gender and smoking was added to the model (AUC 0.82, 95% CI 0.76–0.87, p<0.001). No significant effect modification by gender or smoking was present, as determined by *post hoc* split-file analyses. Salivary *A. actinomycetemcomitans* levels and serum antibodies were almost as efficient in periodontal diagnostics, compared to *P. gingivalis*. Of note, the results of study I should be considered rather preliminary, while they are based on a cross-sectional setup with a fairly small sample and stringent inclusion criteria. On the other hand, this study is, to the best of our knowledge, the only publication in periodontal diagnostics with simultaneous access to accurate quantitative measurements of salivary pathogens and corresponding serum IgA/IgG response. A combination of salivary and serum biomarkers is a promising biomarker in periodontal diagnostics, for example in epidemiologic surveys or as a remedy for other health care providers and more research on this topic is warranted. However, at the dentist's office, this kind of approach is unlikely to supersede the clinical and radiographic oral examinations.

Table 5. Predictive capability of combined salivary pathogen levels and serum antibodies for CGP

Pathogen		AUC	95 % CI	p-value
A. actinomycetemcomitans	Model 1	0.62	0.54–0.70	**0.002**
	Model 2	0.76	0.69–0.82	**<0.001**
	Model 3	0.79	0.73–0.85	**<0.001**
P. gingivalis	Model 1	0.74	0.66–0.81	**<0.001**
	Model 2	0.82	0.76–0.87	**<0.001**
	Model 3	0.84	0.78–0.89	**<0.001**

C-statistics with CGP compared to the rest of the population. Model 1 includes salivary pathogen levels (qPCR, GE/mL) and serum IgA and IgG levels (EU). Model 2 includes additionally age, sex and smoking (never, former or current). Model 3 includes additionally oral hygiene routine obtained from the questionnaires (frequency of brushing and interdental cleaning). CGP, chronic generalized periodontitis (≥ 14 teeth with PPD ≥ 4mm) n=84; AUC, Area under the curve; Ig, immunoglobulin; EU, ELISA units. Bold indicates statistical significance ($p < 0.05$).

As previously discussed (chapter 1.8.1), periodontitis associates with elevated serum antibody levels for periodontal pathogens, and simultaneous presence of high subgingival bacterial levels characterizes an active form of the disease (Study II, Supplementary table I). Using the data acquired from the Parogene population, we explored if any combination of subgingival bacterial level- and/or serum antibody (IgA/IgG) level parameters would be effective in discrimination of different groups of periodontitis (unpublished results). We employed a similar statistical protocol as reported by Persson et al. (2008) (Persson et al. 2008), with species-related variables as continuous parameters. First, we used a Mann-Whitney U test to detect variables associated with active periodontitis. The selection criteria was determined at $p<0.001$ (Persson et al. 2008) or $p<0.25$ level of statistical significance, which has been described as a purposeful cut-off point for variable selection prior to logistic regression (Bursac et al. 2008). Secondly, these variables were inputted in a binary forward (Wald) stepwise logistic regression model, with active periodontitis (Vs. rest of the population) as the dependent variable. *P. gingivalis* serum IgG and subgingival levels of *P. endodontalis* and *T. forsythia* were additive to the model fit. A combination of these variables yielded a good predictor for active periodontitis in ROC analysis (AUC 0.68, SE 0.025, 95%CI 0.64-0.73). In the c-statistic, adding information of age, gender and smoking (never/ever) increased the predictive capability of the combination (AUC 0.79, SE 0.028, 95% CI 0.74-0.85).

We performed the same set of analyses for detection of a combination predicting both history of periodontitis and active periodontitis (unpublished results). The most effective combination was *C. rectus* serum IgA along with subgingival levels of *P. gingivalis* and *T. forsythia* (AUC 0.70, SE 0.03, 95% CI 0.65-0.76), and when this combination was complemented with information of age, gender and smoking, the predictive capability was again increased (AUC 0.82, SE 0.02, 95% CI 0.78-0.87).
The periodontal diagnosis is usually determined by a dentist based on a clinical and radiographic evaluation, but alternative diagnostic measures are needed for other health care personnel and periodontal research.
From these analyses, it is clear that combining oral and systemic pathogen-associated variables produce rather good markers for clinical periodontal disease, as was also evident from Study I. Interestingly, the main determinants were combinations of oral pathogen levels and antibody levels, but not for the same pathogen. We used a combination of salivary and serum samples, but restricting the immunoassay and bacterial quantification to one all-encompassing oral sample—saliva— might result in a more useful periodontal biomarker from a clinical or screening point-of view. Still, some additional conclusions can be drawn from these results. First, any single pathogen, or its immune response, is not likely to represent a sufficient biomarker for periodontitis, while it is a heterogenic inflammatory disease with a polyinfectious etiology. Secondly, results from a majority of studies are much influenced by the preceding hypothesis, for example in selection of studied pathogens, and always represent some degree of "cherry picking", a fallacy of selected attention. This emphasizes the importance of more microbiological and seroepidemiological studies using a vast array of bacterial species.

4.2 Associations between periodontal pathogens, serum antibodies and CAD (I, II, IV)

4.2.1 Oral bacteria elicit a systemic immune response (I, II, IV)

Oral bacteria are known to elicit a humoral immune response, as previously discussed. We have analyzed these antigen-antibody associations in three different substudies (I, II, IV). **Table 6** shows the crude correlation coefficients regarding oral bacterial levels and serum antibodies. Regarding the Health 2000 substudy (I), with 230 dentate Finnish adults, salivary *A. actinomycetemcomitans* and *P.*

gingivalis and corresponding serum IgA/IgG levels had a weak to moderate statistically significant correlation. Also, we found a significant positive association between the salivary pathogen levels and corresponding serum IgA/IgG levels for both species in linear regression analyses (study I), adjusted for age, gender, smoking and stages of periodontitis ($p<0.010$ in all analyses) demonstrating the inevitable immune response following antigen exposure.

In study I, seropositivity (either IgA or IgG) indicated salivary pathogen prevalence with a sensitivity of 41% and 69% and specificity of 81% and 56% for *A. actinomycetemcomitans* or *P. gingivalis*, respectively. These results are in line with the research groups' earlier project (Pussinen et al. 2011), which concluded that salivary prevalence of *A. actinomycetemcomitans* and *P. gingivalis* was the main determinant of corresponding serum antibody response. In that study, bacterial prevalence was determined with conventional PCR in a large population-based subcohort of the Health 2000 survey (N=1,586), while in contrast, we used a smaller subsample of the same cohort with quantitative bacterial determinations and a case-control setup.

Salivary levels of *A. actinomycetemcomitans* and *P. gingivalis* correlated with their corresponding serum IgA/IgG levels in the Health 2000 subsample (**Table 6**). Single copy gene-based qPCR has shown to be accurate and sensitive in detection of these pathogens, with a detection threshold of 8–16 genomes/ml template (Hyvärinen et al. 2009). The correlation coefficients between subgingival bacterial levels and systemic antibody response in the Parogene population (II, IV) were statistically significant only regarding *P. gingivalis* (IgA/IgG), *P. endodontalis* (IgA/IgG), and *P. intermedia* (IgG) (**Table 6**); these antibody levels associated significantly also with subgingival prevalence of the species (Study II, Table 2). Of note, all published results that are based on the Health 2000 study (I) or the Parogene study (II, III, IV) are limited by a cross-sectional design, and no conclusions of direction of events can be drawn.

A majority of the Parogene population had low antibody levels, with a rather skewed distribution, which might partly explain the lack of statistical significance. Subgroup analyses and factorial ANOVA across groups of smoking (never/ever), age (60 or 65 years as threshold) or periodontal status (four groups) did not notably alter the results, which would have proposed interaction effects with these variables. The used DNA-DNA hybridization technique (II, IV) is less sensitive for bacterial detection,

compared to qPCR, and it can be routinely used to detect 10^4 cells of any given species (Socransky et al. 2004) and a threshold of 10^5 has been previously used in the Parogene population for bacterial detection (Mäntylä et al. 2013).

Table 6. Correlation between oral bacterial levels and corresponding serum antibody levels.

	Health 2000 substudy (I)*		Parogene study (II, IV)**	
Pathogen	Serum IgA	Serum IgG	Serum IgA	Serum IgG
	Spearman's ρ		Spearman's ρ	
A. actinomycetemcomitans	**0.27**	**0.31**	-0.02	0.01
P. gingivalis	**0.50**	**0.45**	**0.39**	**0.45**
F. nucleatum			0.01	-0.01
P. endodontalis			**0.12**	**0.13**
T. forsythia			-0.02	0.02
C. rectus			0.05	0.04
P. intermedia			0.08	**0.10**

Nonparametric Spearman's correlation (two-tailed) coefficients calculated with crude linear variables including the whole population. * Salivary bacterial levels measured with qPCR; ** Subgingival pathogen levels were measured with checkerboard DNA-DNA hybridization; Immunoglobulins (Igs) were measured with ELISA. Bold indicates statistical significance ($p<0.05$).

Of note, changing the detection level threshold to 10^5 in Study II did not alter the significance levels for antigen-antibody associations. Haffajee et al. (1995) (Haffajee et al. 1995) noted that the subgingival levels of any pathogen associated weakly with corresponding serum antibodies and speculated that high antibody levels might diminish bacterial load or alternatively the bacteria present in high levels in the subgingival samples might not represent pathogens. The sample collection method might be a biasing factor, while a pooled bacterial sample from the most diseased site in each dental quadrant does not perfectly represent the whole oral microbial burden. Also, local differences in host-evasion mechanisms between bacterial species has been speculated to impact the systemic antibody-levels (Saraiva et al. 2014).

Interpretation of the results presented above is complicated by the fact that we used two different population with different oral samples, as it would have been beneficial to have information on both salivary and subgingival bacterial determinations from the same population. However, a few points should be considered when comparing of immune response to subgingival vs. salivary microbes. Both reflect the oral microbiota, but the subgingival biofilm could be considered a more static microenvironment, which represents a higher concentration of the pathogenic bacterial community. The systemic effects and of the subgingival biofilm, as well as the immune response, are probably mostly spread from the inflamed gingival tissue. Saliva, on the other hand, represents a broader overview of the oral microbiota, and a part of the systemic effects and immune response might be transmitted via the gut. The oral cavity is the primary entry point for the whole gastrointestinal microbiota. It has the highest bacterial diversity but shares a high degree of species similarity when compared to intra-individual bacterial samples from the duodenum, colon and stool (Stearns et al. 2011). However, a metatranscriptomic study showed that even though a subset of oral bacteria routinely survive transit to the gut, they expressed little transcriptional activity (Franzosa et al. 2014). The healthy gut is in constant dynamic interaction with the luminal homeostatic microbiota, but the gut permeability might be increased as a response to e.g. any inflammatory process, diet and stress (Quigley 2016). A pathological bacterial breach in the "leaky gut" induces hyperactivation of the mucosal adaptive immune system and chronic inflammation (Ahmad et al. 2017). Periodontitis is also believed to influence inflammatory bowel disease via systemic inflammation, and thus further impairs the intestinal barrier. These diseases are probably bidirectionally linked and involve a complex interplay between the immune-inflammatory response and the dysbiotic microbiota, modified by environmental and genetic factors. (Lira-Junior & Figueredo 2016). However, the potentially causal relationships between oral microbiota, intestinal dysbiosis, intestinal inflammation and increased intestinal permeability are yet to be determined (Hamilton & Raybould 2016).

Taken together, our results suggest that oral bacteria provoke a humoral immune response. Salivary bacterial levels correlate better with the corresponding serum antibodies than subgingival bacterial levels (**Table 6**). Saliva might better represent the oral microbial load, compared to selected subgingival samples, and a part of the antigen exposure might activate the immune response in the gut via swallowed saliva.

4.2.2 Periodontitis associates with an infectious and immunologic burden (II)

Beck and coworkers have shown a relationship between high serum titers to several oral bacteria and subclinical atherosclerosis, using the Dental ARIC study with serum IgG determinations for approximately N=4,500 subjects and 17 periodontal pathogens (Beck et al. 2005b). One of the aims in this thesis was to apply a more holistic view of aggregate infectious burden, as it has been hypothesized to represent a potential CVD risk factor (Zhu et al. 2000). This kind of approach is justified, bearing in mind that neither is periodontitis a monoinfection, nor has atherosclerotic risk been exclusively linked to a single pathogen. We summed the obtained serum IgA or IgG levels for each of the seven studied periodontal species to acquire a total IgA/IgG burden, which represents the systemic exposure to typical periodontal pathogens (Beck et al. 2005a). The obtained values for "total burden"-variables are not appropriate for subgroup characterization *per se*, but rather facilitate comparison between groups. The main results are displayed in Study II.

The associations between subgingival bacterial abundance and common periodontal parameters in the Parogene population have been reported earlier (Pradhan-Palikhe et al. 2013), and are not thus a part of this thesis. Our results indicate that both history of periodontitis and active periodontitis associate with a higher level of total serum IgA/IgG burden for *A. actinomycetemcomitans, P. gingivalis, P. endodontalis, P. intermedia, T. forsythia, C. rectus* and *F. nucleatum* compared to periodontally healthy controls (Study II, Suppl. 1). The patients with active periodontitis had additionally higher subgingival pathogen burden, contrary to patients with a history of periodontitis, whose subgingival bacterial levels did not notably differ from periodontally healthy controls. These findings comply well with the preceding hypothesis, that the active phase of periodontal disease with increased BOP%, is characterized with increased subgingival load to periodontal pathogens (Socransky & Haffajee 2005), and the humoral immune response remains stable for long (Lakio et al. 2009) — maybe also after remission of disease, suggesting that mere serology marks a history of periodontitis (Papapanou et al. 2004).

Haffajee et al. (1995) were among the first to study patterns of periodontal disease, subgingival pathogen levels and corresponding serum antibodies to 12 bacterial species (Haffajee et al. 1995). It was noted that the most effective parameter in periodontal discrimination was not any single

pathogen, but a finite number of bacterial clusters, with a rather high interindividual variability. They also noted that the most active form of periodontitis is probably defined by simultaneously elevated levels of subgingival pathogen and humoral immune response (Haffajee et al. 1995, Pussinen et al. 2011), which was also evident in the present study.

4.2.3 Is periodontitis linked to a hyperresponsive antibody production?

The consensus report of the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions (Lang et al. 1999) stated that a poor antibody response towards infecting agents associates with generalized aggressive periodontitis. This hypothesis has been criticized with opposing data (Hwang et al. 2014), and further tested with an "infection ratio" variable as a measure for antibody responsiveness for each periodontal pathogen separately. The "infection ratio", introduced by Picolos et al. (2005), was calculated as "subgingival bacterial level"/"serum antibody level" for corresponding pathogen (Picolos et al. 2005). As we also had access to quantitative data on subgingival pathogens (seven species), and their corresponding serum IgA/IgG, we calculated "infection ratio" variables for each pathogen, separately for IgA and IgG antibodies, as previously suggested (unpublished results). We performed a series of Mann-Whitney tests, comparing these infection ratios between various groups of periodontal diagnoses and periodontally healthy controls. Of all tests, only *P. gingivalis* IgG infection ratio was statistically significant ($p=0.006$), as patients with active periodontitis had a lower median value of *P. gingivalis* IgG "infection ratio" compared to controls. Our results are much in line with the original report by Picolos et al., who concluded that there are no conspicuous differences in infection ratios between periodontal subgroups and that this kind of approach in evaluating infection patterns are likely too simplistic and rudimentary (Picolos et al. 2005).

4.2.4 Immune response to periodontal pathogens as a CVD risk factor (II)

One of the main premises of this thesis is the well accepted concept that oral inflammatory disease increases the risk for CVDs independently of potential confounders (Lockhart et al. 2012), and several mechanistic links have been proposed (Schenkein & Loos 2013). Systemic antibodies against oral micro-organisms are probably the best justified measure for systemic exposure, with several reasons explained in a review by Teles & Wang (2011) (Teles & Wang 2011). For example, humoral immune response to a microbe can be interpreted as a proof of its pathogenic potential due to recognition of foreign antigens (Haffajee & Socransky 1994), a vigorous antibody response might reflect the immune responsiveness of the host, which in itself is a CVD risk factor (Mattila et al. 2005), and IgG-response is stable and indicates cumulative exposure, while IgA-response indicates a more recent exposure (Pussinen et al. 2007a).

Using the Parogene population, we applied a binomial logistic regression model to evaluate if total IgA/IgG burden associates with angiographically verified stable CAD or ACS. In preliminary analyses, we noted that even a slight increase in the aggregate serum antibody levels significantly associated with ACS, and therefore used the lowest antibody quartile as the reference. Quartiles 2-4 of total antibody burden associated significantly with ACS in a logistic regression model adjusted with age, gender, smoking (never/ever), dyslipidemia, hypertension, diabetes, body mass index (BMI) and number of teeth present (Odds ratio [OR] 2.0, 95% CI 1.1-3.5, p=0.017 for IgA; OR 1.8, 95% CI 1.0-3.2, p=0.035 for IgG, Study II, table 4). The results were not notably altered when subgingival pathogen burden was added to the model.

Previous reports by Pussinen et al. concluded that high serum antibodies to the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* associate with subclinical, prevalent and future incidence of CHD in a 10-year follow up study including 1023 Finnish men. Highest tertile of *A. actinomycetemcomitans* IgA indicated a Risk ratio (RR) 2.0 (95% CI 1.2-3.3) for CHD-related causes of death, including AMI (Pussinen et al. 2005). They also reported in another longitudinal study of 63 nonfatal AMI patients and matched controls that high levels of serum *P. gingivalis* –specific IgA increases the risk for primary myocardial infarction (OR 2.5, 3.3, 4.0 for quartiles 2-4 respectively) (Pussinen et al. 2004a). A study based on over N=1173 men from the Oslo II study concluded that

serum IgG antibodies against *A. actinomycetemcomitans, P. gingivalis, T. forsythia* and *T. denticola* associated with an approximately 30% increased relative risk for self-reported AMI in a regression model adjusted for typical cardiovascular confounders (Lund Håheim et al. 2008). The report concluded that simultaneous analysis of exposure to multiple periodontal pathogens is more relevant than inspecting any single pathogen, supporting the infectious burden hypothesis (Zhu et al. 2000). The above-mentioned findings are in concordance with our results, where even slightly increased levels of serum IgA/IgG burden for periodontal pathogens indicated elevated risk for ACS.

4.2.5 Immune response — a plausible link between oral pathogen load and CVDs

We have provided evidence that periodontal infections elicit a systemic immune response, which associates with CVD phenotypes. Past publications on the same topic are mainly based on in vitro studies, animal models and epidemiologic surveys. The mechanistic links seem credible, but from a statistical point of view, these are prone to some bias due to multiple comparisons error. To our knowledge, there are no past studies where immunologic burden to periodontal pathogens are considered mediators between oral pathogen burden and CAD in a formal statistical setup. We studied the link between total subgingival pathogen burden and stable CAD or ACS with a statistical test of mediation using the Parogene population (in press). Bootstrapping is often considered the preferable method, while it is suitable for small sample sizes, has the highest power, best type I error control, does not require normal distributions and accounts for multiple comparisons error. Hayes' PROCESS macro (model 4) for SPSS™ (version 24; IBM Corp, Armonk, NY, USA) was applied for simple bootstrap mediation analysis, where Stable CAD or ACS was used as the outcome, total subgingival bacterial burden was used as the independent variable, and total IgA- or IgG-burden as the mediator.

A statistically significant indirect effect of the independent variable on the outcome, with 95% CI >0 or <0, was considered sufficient to support a claim of mediation (Hayes & Rockwood 2016). Using these strict criteria, no mediation of effects was evident in the whole population. Age is a significant effect modifier in the humoral immune response to oral bacteria (Papapanou et al. 2000, Vlachojannis et al. 2010), and the Parogene cohort is mainly comprised of older adults. Therefore, we performed similar *post hoc* tests for subjects <65 years of age, while that threshold was the population median.

Subgingival pathogen burden associated significantly with corresponding serum IgA burden (a=0.158, SE=0.070, p=0.025, CI 0.020-0.296), which in turn associated with ACS (b=0.359, SE=0.177, p=0.042, CI=0.012-0.706). There was a significant indirect effect of subgingival burden on ACS through serum IgA burden, ab=0.057, BCa CI (0.006-0.168). Both the total- (c=0.061) and direct effects (c'=0.061) of subgingival bacterial burden on ACS were non-significant. With these analyses, we showed that the subgingival burden for seven periodontal pathogens have a significant indirect effect on ACS through systemic IgA burden (**Figure 6**). These findings support the hypothesis that the association between periodontitis and CVD is partly mediated by the immunologic response for periodontal pathogens.

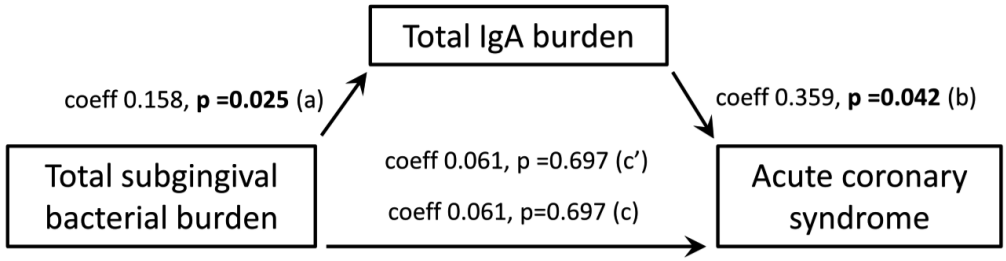

Figure 6. Serum IgA response mediates effects between periodontal pathogen burden and ACS.

Formal test of mediation performed with Hayes' PROCESS macro for SPSS™ (model 4). Parogene cohort was confined into N=173 patients <65 years of age. No coronary artery disease (reference), n=88; ACS, n=85. Subgingival bacterial levels and corresponding serum IgA levels for *A. actinomycetemcomitans, P. gingivalis, P. endodontalis, P. intermedia, T. forsythia, C. rectus* and *F. nucleatum* were summed and standardized prior to analysis. ACS, Acute Coronary Syndrome.

4.3 LPS as a potential mediator between oral infections and CAD (III, IV)

LPS, a proinflammatory molecule and a principal membrane structure of gram-negative bacteria, has been proposed as one of the molecules that mechanistically links oral infectious diseases and atherogenesis, as previously discussed (Chapter 1.7). Systemic LPS, endotoxemia, is known to contribute to low grade systemic inflammation, which is a risk factor for a wide range of chronic conditions, such as the MetS, non-alcoholic fatty liver disease, type 2 diabetes and CVD (Minihane et

al. 2015). This thesis focuses on cardiometabolic disorders, primarily CVD-subtypes (II, III, IV, V) and diabetes (V) as the end-points.

We aimed to study how periodontal pathogen burden, salivary and serum levels of LPS interrelates with AP (IV), periodontitis (III) and CAD. Our data from the Parogene population was suitable for this purpose, while it includes quantitative data on subgingival bacteria (70 species, Study III, suppl. table I), salivary and serum LPS activity, extensive clinical and radiographic oral examinations and accurate angiographically verified coronary status. However, it is mainly limited to a restricted population of Finnish adults with an initial indication for coronary angiography. Therefore, the obtained results might not be applicable to the general population due to selection and Neyman's bias.

In general, LPS concentrations were approximately 10^5 higher in saliva compared to serum (Study III, table 2), which is not surprising considering that the oral cavity exhibits a vast microbiota. Of the studied oral and coronary parameters, only ABL associated positively with increased salivary LPS levels, although *post hoc* pairwise comparison between groups with a Bonferroni correction did not result in statistically significant differences. These findings are somewhat contradictory to the ones by Lappin et al. (2011) (Lappin et al. 2011), who concluded that concentrations of salivary TLR4 stimulants (mainly LPS) are higher in patients with periodontitis. However, their method was fairly different from ours, as Lappin et al. used a bioassay based upon the measurement of NF-κB-dependent reporter activation in TLR-deficient human embryonic kidney 293 cells transfected to express human TLRs (Lappin et al. 2011). That assay detects host cell sensitive TLR ligands, such as LPS, lipopeptides and peptidoglycan (Munford 2016). The LAL assay used in our studies detects LPS activity, by recognition of the lipid-A moiety of the LPS molecule, which initiates the Limulus amebocyte clotting cascade (Munford 2016).

The LAL assay is the most widely used and most sensitive assay for LPS activity (Manco et al. 2010) although mere LPS activity measurements have been criticized as LPS of some gram-negative oral organisms have been shown to be antagonists, rather than agonists, of human TLR4, leading to differing biological effects (Yoshimura et al. 2002). Nonetheless, clinical periodontitis is not systematically associated with higher abundance of gram-negative species, which would intuitively be the main determinant for salivary LPS levels, as discussed earlier. LPS activity in saliva is rarely

measured, and requires more research. For instance, it is largely unknown how the salivary matrix, including activators and inhibitors of LPS activity, affects the LAL assay.

4.3.1 Does apical periodontitis induce endotoxemia? (IV)

AP represents another form of a chronic oral infection with potentially deleterious systemic effects, as discussed in chapter 1.5.3. Potential direct associations between AP and coronary outcomes are explained in detail in chapter 4.4. Several inflammatory markers have been shown to be elevated in patients with AP (Gomes et al. 2013) and mice with an endodontic infection have perceivably higher levels of serum LPS (Furusho et al. 2013). To the best of our knowledge, no previous studies have been published regarding AP and endotoxemia in humans. In study IV, we analyzed the associations between AP and CAD, and considered the humoral immune response to *P. endodontalis,* or serum LPS levels as potential mediators of effect.

In study IV, serum LPS levels only differed between groups of cardiological outcome, but the statistical effect was not significantly modified by subgingival *P. endodontalis* levels, serum IgA/IgG against it, or score of AP (Two-way ANOVA, $p>0.05$). We performed additional analyses with various AP related parameters as the independent variable (total number of widened periapical spaces, total number of apical rarefactions, our score for periapical pathosis, number of root canal treated teeth and untreated teeth with AP) and salivary/serum levels of LPS as the dependent factor (unpublished results). In this population, no statistically significant effects of AP on LPS levels were detected. This might be due to the fact that chronic AP restricts spread of infection and endodontic instrumentation provokes bacteremia in very low levels (Reis et al. 2016).

Noteworthily, our lack of information on the endodontic microbiota is a bias. Similar studies with proper characterization of the root canal biofilm and systemic immunological response to it are warranted. However, our hypothesis that AP might increase serum levels of LPS is still justified, as teeth with AP have shown to have high endodontic LPS concentrations, (Gomes et al. 2012a) and endodontic procedures might cause bacteremia in approximately 30% of cases (Olsen 2008). To conclude, further studies are needed to assess if AP *per se* causes clinically significant endotoxemia

4.3.2 Subgingival bacterial abundance affects salivary LPS activity

We performed a series of age and gender adjusted linear regression models for the whole population to evaluate if the subgingival levels for any of the 70 studied species associated with salivary LPS levels. We found a significant association between saliva LPS levels and *P. micra, P. intermedia, C. rectus, F. nucleatum* (four subspecies), *Pseudomonas aeruginosa, A. actinomycetemcomitans, P. gingivalis* and *Capnocytophaga gingivalis* (Study III, Table 3). Interestingly, the main determinants for salivary LPS were oral gram-negative species and known pathogens for periodontitis, although preceding assumptions were not made. A combined cluster of these species explained 3.2% of the variation in salivary LPS levels (R^2=0.032 in an unadjusted linear regression model).

Using the similar model, we did not detect any statistically significant positive correlations between subgingival microbe levels and serum LPS. Kallio et al. (2015) (Kallio et al. 2015) showed in a large longitudinal population based study that endotoxemia is strongly associated with cardiometabolic disorders. Therefore, it would have been interesting to analyze if subgingival microbes have a direct effect on serum LPS levels in metabolically healthy subjects. However, the Parogene population is not well suitable for that purpose, as a majority represented metabolically diseased subjects. A total of 80% suffered from dyslipidemia (statin medication), 64% were hypertonic and 30% were obese (BMI ≥ 30), all being criteria for MetS (Alberti et al. 2009). As transmucosal transport of LPS is promoted by a high-fat diet, this factor would also be useful to consider (Piya et al. 2013). Limiting the analysis to metabolically healthy subject would have diminished the statistical power, and we therefore leave it as a recommendation for future studies. Nevertheless, we showed a significant correlation between salivary and serum LPS (Pearson's r=0.092, p=0.046) which was enhanced in a subpopulation of active periodontitis (Pearson's r=0.222, p<0.001). For patients with active periodontitis, 4.9% of the serum LPS variation was explainable by saliva LPS. As virtually all of the salivary LPS is originated from local microbiota, MetS was not considered a confounding factor in these analyses.

4.3.3 Endotoxemia — a plausible link between oral pathogen load and CVDs

Circulating LPS is known to mediate atherogenic processes e.g. by promoting vascular inflammation (Stoll et al. 2004). Several molecular mechanisms behind this link have recently been reviewed (Jiang et al. 2017) and endotoxemia has been shown to associate with incident CVD events in a prospective observational study with 10 years of follow-up time (Pussinen et al. 2007b). We analyzed the association between serum LPS tertiles and angiographically verified stable CAD and ACS in three different statistical models with additive adjustments for potential confounders (**Figure 7**). We found that high levels (3rd tertile) of serum LPS associated with stable CAD independently of CAD risk factors (OR 2.0, 95%CI 1.0-3.8, p=0.039), supporting the above-mentioned premise. The results reported in study III indicate that LPS is a possible mediator between periodontitis and CAD (Lockhart et al. 2012). However, the chain of analyses is prone to multiple comparisons error, and we performed additional *post hoc* tests to further evaluate if the claim supports true mediation (unpublished results).

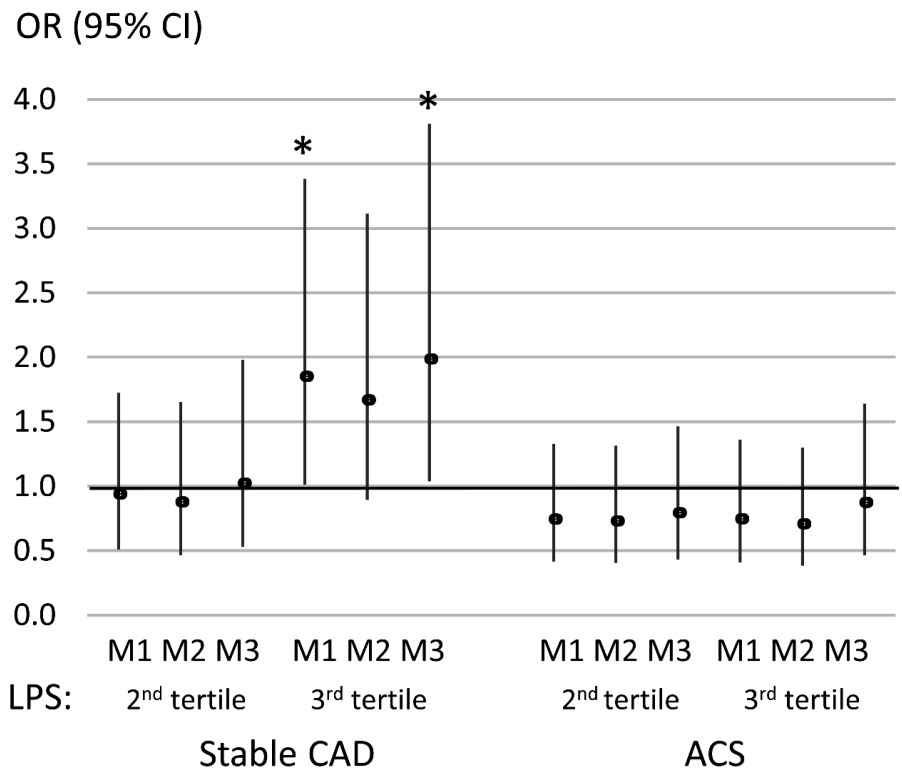

Figure 7. The association between serum LPS tertiles and CAD/ACS.

Multinomial logistic regression with first serum LPS tertile used as reference. Model 1 adjusted for age and gender. Model 2 additionally adjusted for dyslipidemia (yes/no), hypertension (yes/no), smoking (never/ever). Model 3 additionally adjusted for periodontitis (4 groups). CAD, stable coronary artery disease; ACS, acute coronary syndrome.

As in chapter 4.2.5, we used Haye's PROCESS macro (model 6) for SPSS™ as a formal test of mediation, where Stable CAD was used as the outcome, the cluster of 11 bacteria that associated with salivary LPS was used as the independent variable, salivary LPS levels as the 1st mediator and serum LPS levels as the 2nd mediator. The conceptual diagram is depicted below (**Figure 8**). In this model, there was a significant indirect effect (=0.0006, BCa CI 0.0003-0.0024) of subgingival burden on stable CAD through saliva- and serum LPS levels. This finding supports a claim of mediation of the subgingival bacterial abundance on stable CAD through salivary- and serum LPS (Hayes & Rockwood 2016).

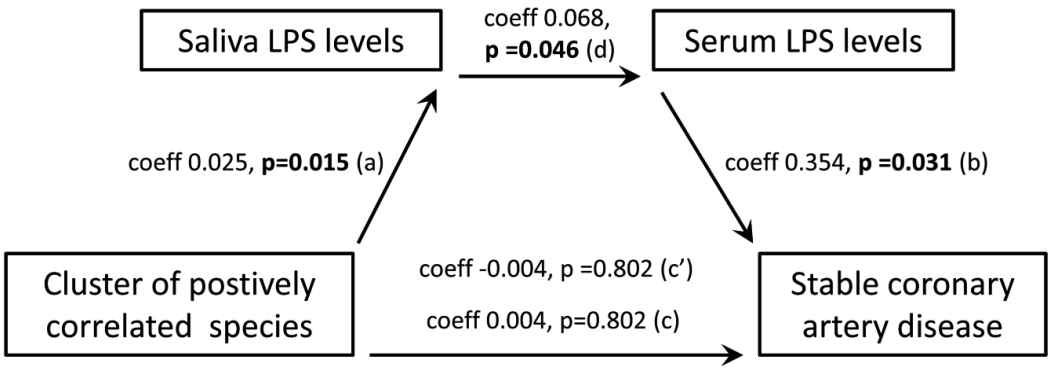

Figure 8. Salivary and serum LPS levels mediates effects between periodontal pathogen burden and Stable CAD.

Formal test of mediation performed with Hayes' PROCESS macro for SPSS™ (model 6). No CAD (reference), n=123; Stable CAD, n=184. Subgingival bacterial levels for *P. micra, P. intermedia, C. rectus, F. nucleatum* (four subspecies), *P. aeruginosa, A. actinomycetemcomitans, P. gingivalis* and *C. gingivalis* were summed and standardized prior to analysis. LPS levels (EU/ml) were available as continuous variables. CAD, Coronary Artery Disease.

4.4 Apical periodontitis and CAD (IV)

As discussed in the introduction (chapter 1.10), there is some evidence to date supporting the hypothesis that AP constitutes an increased risk for CVDs. Compared to marginal periodontitis, this is a fairly new line of research, initiated by Frisk et al. in 2003 (Frisk et al. 2003), although AP was considered as a potential CVD-risk factor as a part of the "total dental index" introduced by Mattila et al. already in 1989 (Mattila et al. 1989). Recently, two noteworthy systematic reviews have attempted to draw conclusions on the associations between AP and CVD, based on a rather few observational studies (Khalighinejad et al. 2016, Berlin-Broner et al. 2016). Both concluded that there may be a confounder independent association between AP and CVD, with a low-moderate degree of evidence, but any claims of causality are unwarranted and further research is needed.

In study IV we analyzed the associations between radiographically determined AP and angiographically verified CAD outcomes in a cross-sectional setup using the Parogene cohort. Our study should be regarded complementary to the ones reviewed by Khalighinejad et al.

(2016)(Khalighinejad et al. 2016) and Berlin-Broner et al. (2016) (Berlin-Broner et al. 2016), as their literature search was limited to a time prior to publication of Study IV.

From Study IV, Table 1, it is clear that oral infections were fairly prevalent in the Parogene population. Among all dentate subjects, 48% had ≥1 carious lesion, 54% had ≥1 widened periapical spaces, 24% had ≥1 apical rarefactions and 70% had ≥1 RCF, indicating a previous need for endodontic therapy. Within the subgroup with RCF (n=341) 79% had at least one RCF of poor quality. In contrast to a majority of past studies, we combined information on widened periapical spaces and apical rarefactions to produce for each patient a 3 staged AP-score, which reflects the continuum of the infectious endodontic status. Common demographic and cardiological characteristics for the whole population and different scores of AP are displayed on Study IV, Table 3. The AP-score associated with BOP ($p<0.001$) and ABL ($p=0.016$), indicating that same subjects with endodontic infections more often suffered simultaneously from periodontal disease. This finding is not surprising, as both diseases are at least partly caused by plaque accumulation due to insufficient oral health routines. Therefore, marginal periodontitis is a confounder in the association between AP and CVD, and perhaps *vice versa*, a fact that has often been overlooked (Gomes et al. 2013).

We used a multinomial regression model to analyze the associations between AP-parameters and coronary outcome, using "no CAD" (n=123) as the reference (Study IV, Table 2). The independent variables of interest were having widened periapical spaces (n=234), having ≥ 1 apical rarefactions (n=108), having ≥1 apical rarefactions in teeth without RCF (n=57) and highest AP score (n=76). The models were adjusted with age, gender, smoking (never/ever), diabetes, BMI, ABL (none vs. mild to severe) and number of teeth present. The main results are illustrated as OR (95% CI) in **Figure 9**. Of note, all analyses were repeated with edentulous subjects excluded, and the results were not notably altered.

In this study, we demonstrated a confounder adjusted association between AP and CAD, especially ACS, supporting the preceding hypothesis. Having widened ≥1 periapical spaces associated with stable CAD (OR 1.94, 95% CI 1.13-3.32, $p=0.016$) and highest AP-score associated with ACS (OR 2.46, 95% 1.09-5.54, $p=0.030$). This association was especially notable in subjects with ≥1 teeth with apical

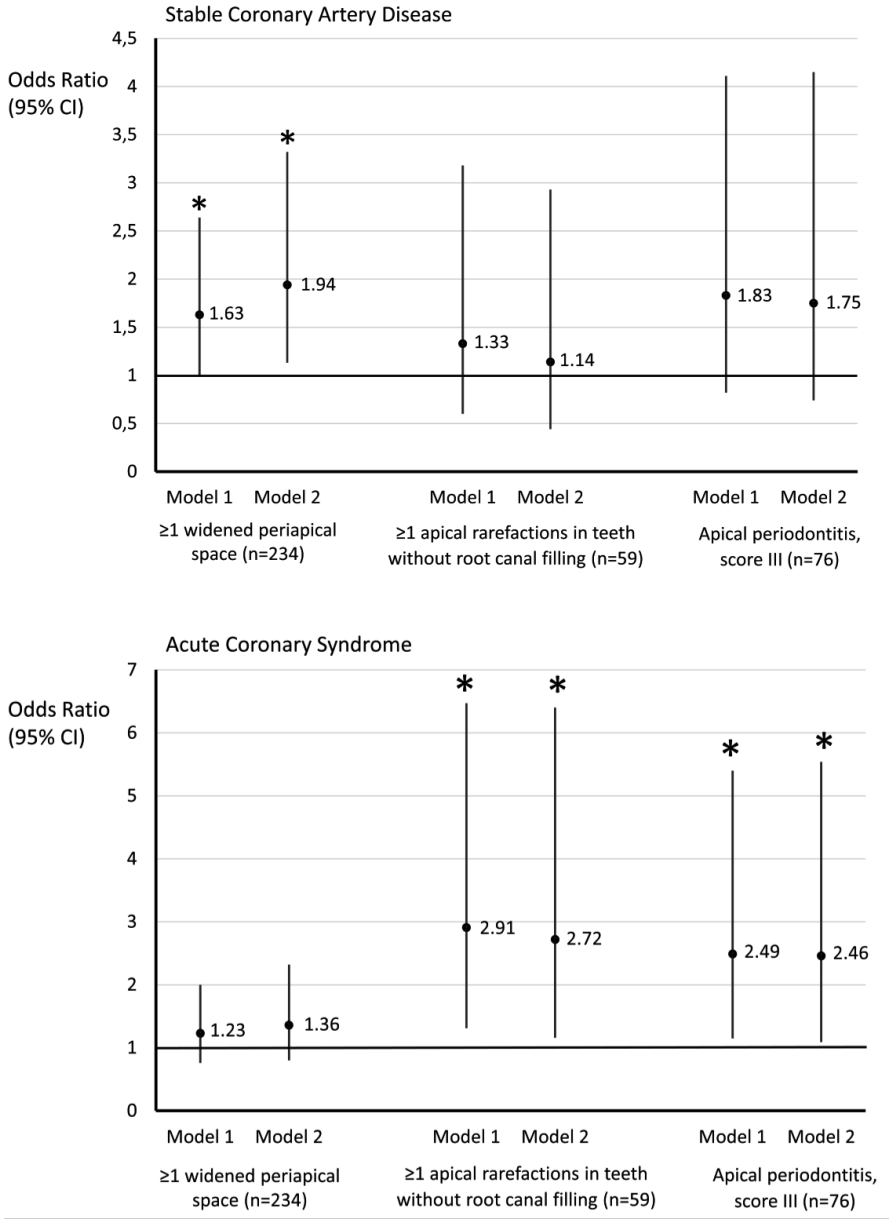

Figure 9. The association between apical periodontitis and Stable CAD/ACS.

Multinomial logistic regression with "no CAD" (n=123) used as the reference; Model 1 adjusted with age and gender; Model 2 additionally adjusted with smoking (never/ever), diabetes, body mass index (BMI), ABL and number of teeth present. CAD, Coronary Artery Disease; ACS, Acute coronary syndrome. *p<0.05.

rarefactions but no RCF (n=59, OR 2.72, 95% CI 1.16-6.40, p=0.022). Of note, similar analyses for AP-teeth with RCF did not result in statistically significant associations, suggesting that root canal treatment might attenuate some of the deleterious effect. Possible reasons for this speculation is that treated teeth might leave radiolucent and benign scar-tissue (Garcia et al. 2007), even though the disease would have been treated, or alternatively the slowly healing AP lesions might be in a healing phase (Zhang et al. 2015), which was not possible to control for in a cross-sectional study. Petersen et al. (2014) noted a similar protective effect of root canal treatment on atherosclerosis, but further studies are needed to test this hypothesis (Petersen et al. 2014).

Referring to the findings in Study IV, it could be hypothesized that the potentially harmful systemic effects of teeth with AP are emphasized in teeth without RCF, a factor that we suggest should be accounted for in future studies. Recently, Meurman et al. (2017) proposed that having 1≥ teeth with RCF might associate with lower risk for prevalent CAD and also lower CVD-mortality incidence (Meurman et al. 2017). The mechanism behind this finding was thought to depend on a decrease in systemic inflammatory mediators following root canal treatment, and largely assumes that patients in their cohort without RCF have a worse oral health, *i.e.* having untreated oral inflammations. In the Parogene cohort, we had access to 7 years of follow-up information on CVD mortality (n=35, ICD-10 codes I00 through I99) and all-cause mortality (n=55).

In the binomial logistic regression analysis (adjusted for age, gender, smoking [never/ever], dyslipidemia [no/yes], diabetes, hypertension [no/yes] and periodontitis [≥1 pocket with PPD ≥ 6mm), only age (OR 1.0, 95% CI 1.0-1.1, p=0.003), male gender (OR 3.3, 95% CI 2.1-5.2, p<0.001) and dyslipidemia (OR 1.9, 95% CI 1.1-3.2, p=0.014) associated with prevalent CAD, while having 1≥ teeth with RCF was not a significant predictor. In the Cox regression model with similar adjustments, age (HR 1.1, 95% CI 1.0-1.1, p=0.001) and smoking (HR 2.6, 95% CI 1.2-5.5, p=0.016) were the only statistically significant predictors for incident CVD-mortality. Having ≥1 RCF at baseline showed some protective effects regarding CVD-mortality with borderline significance (HR 0.529, 95% CI 0.264-1.06, p=0.073), when periodontitis was removed from the model. Having ≥1 RCF at baseline had a statistically significant protective effect for all-cause mortality (HR 0.54, 95% CI 0.31-0.95, p=0.033), but only when periodontitis was excluded from the adjustments. The main limitation in these analyses was a low mortality rate, which is likely due to a shorter follow-up time. To conclude, our

preliminary results support the hypothesis that RCF may be associated with reduced risk for death, even though the detected associations were influenced by effect modifiers, such as periodontitis. RCFs might reflect a healthy lifestyle, which must be considered a confounder, and these results must be interpreted with caution.

4.5 Missing teeth as a risk factor for CVD, diabetes and death (V)

As discussed in the introduction (chapter 1.6), missing teeth is *per se* not a proof of oral infectious disease. However, it seems safe to claim that advanced tooth loss is unlikely to reflect anything other than a history of caries and/or periodontitis in the general population, considering that these are the main reasons for tooth loss worldwide (Reich & Hiller 1993, Phipps & Stevens 1995). Therefore, the number of missing teeth might reflect an accumulation of oral inflammatory burden, with sequential systemic effects (Mattila et al. 2002). In study V, we investigated the power of missing teeth in predicting incident CVD—namely, CHD events, AMI, and stroke, as well as incident diabetes and all-cause death. Our prospective population based cohort study utilized 13 years of complete follow-up data of 8,446 subjects and included information on an extensive set of relevant confounders. As many past studies mainly used mortality rates as the main outcome (Ragnarsson et al. 2004, Cabrera et al. 2005, Polzer et al. 2012, Watt et al. 2012), we were the first to report disease incidence in a large population based prospective cohort.

We divided the population into five groups, depending on how many teeth were missing at baseline, assuming that a full dentition is composed of 32 teeth: 0-1 (n=2,461), 2-4 (n=1,532), 5-8 (n=883), 9-31 (1,813), and 32 (n=940) missing teeth, resulting in a total N=7,629 subjects with information on missing teeth and all relevant confounders. The first group was considered healthy controls, and the second group might largely represent subjects without third molars present. The group with 5-8 missing teeth may have additional involvement of caries, periodontitis, or extractions due to orthodontic reasons. The group with advanced tooth loss (9-31) was likely to have current or past periodontitis or severe dental caries with following endodontic infections. Edentulism represent an interesting end point for oral diseases, as all signs of active oral infections are gone but the systemic effects might be irreversible and evident— “the damage is done” (Hujoel et al. 2001a). Two

limitations in study (V) are worth mentioning; the reasons for past tooth extractions were unknown and we did not have follow-up information on the number of missing teeth, as they were counted only at baseline.

The subjects in higher missing teeth groups were generally older (p=0.001), male (p=0.006) less educated (years of education, p=0.006) more obese (BMI, p=0.005), had higher concentrations of circulating triglycerides (p=0.031), CRP (p=0.003) and lower concentrations of HDL cholesterol (p<0.001) (Study V, Table 1). They also had more often prevalent diabetes (p=0.005) or a family history of diabetes (p=0.029) or AMI (p=0.029). These are known risk factors for cardiometabolic disorders, and therefore considered confounders in our study. Subjects with a history of CVDs or diabetes were excluded from the respective follow-up analyses. Cox regression models were performed and adjusted for all the clinically relevant confounders, which are in concordance with the current general cardiovascular risk profile presented in the Framingham Heart Study (for CVD endpoints) (D'Agostino RB et al. 2008) or the established Diabetes Risk Score (for diabetes) (Lindström & Tuomilehto 2003). The acquired survival plots and statistically significant hazard ratios are displayed in **Figure 10**, which shows that the number of missing teeth at baseline associates with incident CVD, CHD events, AMI, diabetes and all-cause mortality even after extensive adjustment for confounders. For example, having ≥5 teeth missing was associated with 60% to 140% increased hazard for incident CHD events ($p < 0.020$) and AMI ($p < 0.010$).

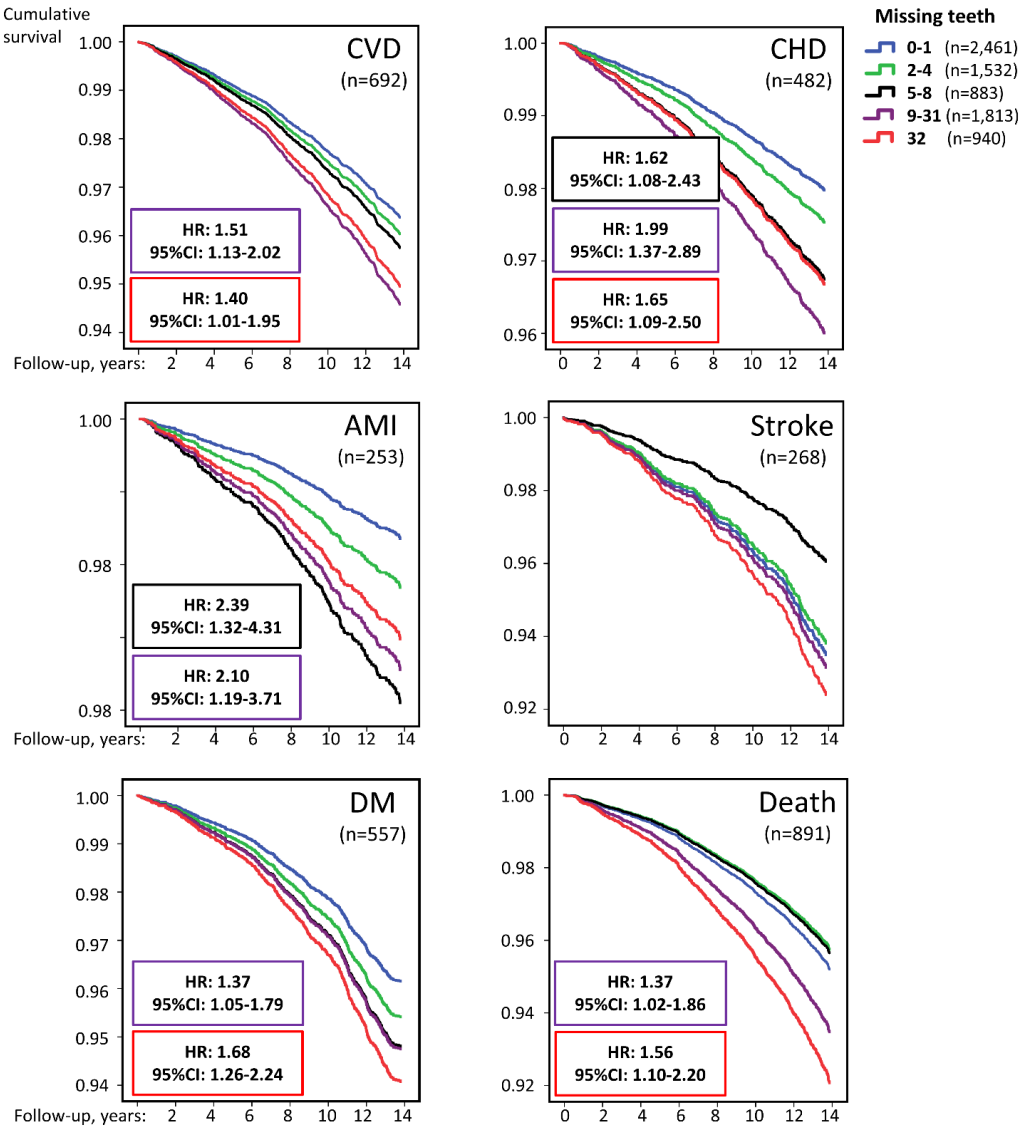

Figure 10: Cumulative survival plots and statistically significant hazard ratios for CVD-outcomes, diabetes and all-cause death across missing teeth groups.

All Cox regression analyses were adjusted for age, sex, smoking (yes/no), and a geographic variable (east/west). CVD outcomes were additionally adjusted for systolic blood pressure, blood pressure treatment (medication within 0 and 7 d, yes/no), total cholesterol (log), HDL cholesterol (log), education (3 categories), and existing diabetes. Diabetes outcome was additionally adjusted for existing CVD. Death as the outcome was additionally adjusted for body mass index, physical inactivity, parent with diabetes, and C-reactive protein (log). CVD, cardiovascular disease; CHD, coronary heart disease; AMI, acute myocardial infarction; CI, confidence interval; HR, hazard ratio.

With the preceding findings, we evaluated if information on missing teeth had an additive effect on the existing prediction models for CVD, diabetes and death. We tested the discriminative ability of the model with and without information on missing teeth using the C-index, the NRI and integrated discrimination improvement statistics (Study V, table 4). The difference in c-statistics was statistically significant regarding all-cause mortality (0.0027, p=0.010), suggesting that missing teeth could be added into existing cardiovascular risk profiles as an additional risk factor. All models were properly calibrated for absolute prediction (Hosmer-Lemeshow test, p>0.05).

Only a few studies on missing teeth have specifically used AMI as one of the endpoints. Tooth loss was concluded to be independently associated with AMI in cross-sectional analyses (Holmlund et al. 2006, Gorski et al. 2016), but no statistically significant association was found in a Japanese 5-year follow up study with 3,081 participants (Noguchi et al. 2015). However, in that study the AMI incidence was low (n=17), which raises concerns of statistical power in the multiadjusted regression models. Therefore, more well-designed prospective studies are required to confirm the hypothesis.

Diabetes and periodontitis are believed to have a bidirectional association. Diabetes leads to a hyperinflammatory response to the periodontal bacterial challenge and hinders tissue repair; mechanisms that are thought to depend on impaired neutrophil function, advanced glycation end products and their receptors. The systemic inflammation caused by periodontitis is believed to promote insulin resistance, adversely affect glycemic control and therefore contribute to development of diabetes (Lalla & Papapanou 2011). A review by Borgnakke et al. (2013) suggests that patient with periodontitis have a greater risk for poor glycemic control, and prevalent diabetes patients have a greater risk for development of diabetic complications, but the level of evidence was deemed scarce (Borgnakke et al. 2013). Moreover, a recent longitudinal study by Winning et al. (2017) concluded that patients with periodontitis patients had a significantly increased risk for incident type-2 diabetes (n=1,331 dentate men aged 58-72 years, median follow-up time 7.8 years) (Winning et al. 2017).

A confounder adjusted association between missing teeth and diabetes has been reported in the US NHANES 2003-2004 (n=2,508) (Patel et al. 2013) and the Hispanic Community Health Study/Study of Latinos (n=15,945) cohorts (Greenblatt et al. 2016). However, these studies used a reverse

hypothesis, using tooth loss as the proposed outcome. Considering that they were cross-sectional in design, bias by reverse causation cannot be ruled out. Demmer et al. (2008) (Demmer et al. 2008) reported that subjects having 25-31 missing teeth had an OR of 1.70 for incident diabetes compared to subjects missing 0–8 teeth at baseline ($p<0.05$) in a prospective study design using 9,296 non-diabetic participants from the NHANES I cohort (mean follow-up time 17 years). Altogether, these reports support our findings that tooth loss ought to be considered a risk factor for incident diabetes.

Solid evidence has emerged that missing teeth is a risk factor for all-cause mortality, supporting the findings from study V. This association was evident in a large cross sectional study with N = 275,424 participants (Wiener & Sambamoorthi 2014) and further strengthened in a prospective study with 24 years of follow-up (Cabrera et al. 2005). A few noteworthy studies have been published in the time between study V and outlining of this thesis. In a Taiwanese retrospective cohort study, 55,651 patients ≥55 years of age were recruited for an annual physical examination during which number of teeth were recorded along with relevant confounding variables (Hu et al. 2015). A total of 3,530 deaths were recorded from national death files during the 6-year follow-up period (mean 3.0 years, SD 1.8). After adjustment for all confounders, the hazard ratios (HRs) of all-cause mortality in participants with no teeth, 1-9 teeth, and 10-19 teeth present were 1.36 (95% CI 1.15-1.61), 1.24 (95% CI 1.08-1.42), and 1.19 (95% CI 1.09-1.31), respectively.

Another large-scale questionnaire-based cohort study was conducted in Australia, where self-reported information on number of teeth was available from 172,630 participants (45-75 years of age), and death incidence was gathered from national registers (median follow-up 3.9 years) (Joshy et al. 2016). Their multiadjusted Cox regression models showed that having <20 teeth left associated with increased death risk, for example in the group with 1-9 teeth present the HR was 1.67 (95% CI 1.45-1.93) compared to the reference group with ≥20 teeth present. In that study, adjustment with covariates was not as extensive as in study V, as no clinical health evaluations with blood samples were made. However, the mere size of the cohort renders the results compelling. The fact that the number of teeth present was self-reported was hardly a bias, considering the relative ease in collecting that information. Moreover, a recent study, based on the STABILITY cohort, including 15,456 patients with stable CAD from 39 countries and a median follow-up time of 3.7 years,

concluded that self-reported tooth loss associated linearly with CVD death, stroke and all-cause mortality (Vedin et al. 2015).

To conclude, compelling evidence has emerged that a reduced dentition associates with a variety of chronic non-communicable diseases and ultimately to an increased mortality risk. We showed that the number of missing teeth may indicate an increased risk for CVD, diabetes, and all-cause mortality, and it could be added into existing cardiovascular risk profiles as an additional risk factor. Criticism is usually raised on the fact that mechanisms behind this association are vaguely described and speculative, considering that there is usually limited information on the reason for tooth loss. The explanation for the observed associations might be systemic inflammation, nutritional compromise, lifestyle factors (such as smoking), association with cognitive decline or other social measures for quality-of-life. However, tooth loss could holistically reflect total stress experienced during a lifetime, involving social, emotional, economic, medical, educational and psychological aspects (Friedman & Lamster 2016). Therefore, significant efforts should be made in healthcare for retention of a healthy natural dentition and the number of missing teeth is an easily collectible measure for detection of patients in risk for shortened life expectancy.

5 Summary and conclusions

Oral infections, such as marginal periodontitis and AP, are among the world's most common infectious diseases. Deciphering the associations between these chronic infections and cardiometabolic diseases is of paramount importance from a public health perspective, considering that chronic noncommunicable diseases, mainly CVD and its complications, are the leading cause of death globally.

The key findings of this thesis are summarized on **Table 7**. We provided strengthening evidence that oral bacterial levels are linked to a systemic immune response (I, II, IV). Saliva is a practical oral sample, as it is easily collectible and provides an overview of the oral microbiota, with pathogen levels that correlate with the corresponding humoral immune response.

Table 7. Key findings of the thesis

Aim	Result
I	• A combination of oral microbiological data and the humoral immune response, especially regarding *P. gingivalis,* discriminated patients with periodontitis from healthy controls.
II	• Elevated serum antibody levels for seven periodontal pathogens indicated past or present periodontitis, while patients with active periodontitis had simultaneously high subgingival bacterial levels. • Increased levels of serum antibodies for periodontal pathogens associated with ACS.
III	• The subgingival levels of several gram negative bacteria, most of which were known periodontal pathogens, contributed to the salivary LPS levels. • Salivary LPS had a mild correlation with endotoxemia. The correlation was more apparent in subjects with active periodontitis. • Endotoxemia associated with CAD.
IV	• AP associated with CAD, especially ACS.
V	• The number of missing teeth associated with incident CVD, diabetes and all-cause mortality. • Information on missing teeth improved the established risk models.

ACS, acute coronary syndrome; LPS, lipopolysaccharide; CAD, coronary artery disease; AP, apical periodontitis, CVD, cardiovascular disease.

Combining information on oral periodontal pathogens and corresponding serum antibodies might be useful as biomarkers in periodontal diagnostics, especially in situations where clinical and radiographic oral evaluation is not possible, such as in epidemiologic surveys and at general medical practitioners' offices. The most promising single pathogen is *P. gingivalis* (I), a keystone pathogen in periodontal etiology, although we promote a more holistic view of the rather heterogenic oral microbiota (II). In high throughput screening with more advanced technologies than qPCR or ELISA, combining bacterial- and host-derived primers or antigens should not be a problem.

The serum antibody levels for periodontal pathogens are proposed to be a useful marker for the systemic exposure to oral infections. An aggregate measure for serum antibody levels is a justified strategy for evaluating the well-established association between periodontitis and CVDs, considering that periodontitis is a multifactorial infectious disease with several putative pathogens, which promote atherogenesis by dissemination of bacteria, their by-products, increased transcription of proinflammatory mediators or induced production of detrimental cross-reactive antibodies.

Patients with active periodontitis had higher subgingival abundance of *A. actinomycetemcomitans, P. gingivalis, P. endodontalis, P. intermedia, T. forsythia, C. rectus* and *F. nucleatum*— seven well known periodontal pathogens— and simultaneously higher serum antibody levels for these species (II). Patients with a history of periodontitis had lower subgingival bacterial levels, although the immunological burden was evident, reflecting a history of infectious exposure with potentially persistent deleterious systemic effects. Elevated serum antibody levels to the aforementioned periodontal pathogens were associated with ACS, supporting the hypothesis that the relationship between periodontitis and CVDs is partly mediated by the immunologic responses to periodontal bacteria (II). The formal statistical tests presented in this thesis provides novel proof that immunologic burden mediates effects of oral pathogen load on ACS.

We are the first to simultaneously study oral bacterial quantities, LPS activity in saliva and serum and high quality coronary diagnosis. We showed that the subgingival levels of several microbial species, most of which were oral gram-negative species or known periodontal pathogens, associated with increased salivary LPS levels. Salivary LPS levels correlated with endotoxemia, especially in patients with active periodontitis. LPS is a well-known pro-inflammatory molecule with proatherogenic

properties, and our study provided new evidence that it might be one of the key mediators of the association between periodontitis and CAD (III). This conclusion was further strengthened by results from a statistical test of mediation.

Even though much is known to date on the association between marginal periodontitis and CVDs, AP has attained much less attention, even though it constitutes another very common chronic and often asymptomatic oral inflammatory disease with similar disseminating systemic effects as marginal periodontitis. In our highly topical study, we demonstrated a confounder-independent association between AP and CAD, which was especially notable with ACS as the outcome (IV).

The number of missing teeth is a crude reflection of the past or present oral infectious status. We showed that missing teeth associates with incident CVD, diabetes or death in a well-designed prospective study (V). Importantly, adding missing teeth to the established risk model helped to reclassify patients with moderate death risk more accurately. The number of missing teeth is a practical marker for crude evaluation of cardiometabolic risk, for example at any health care practitioners' office, and it has potential as a self-report variable in large surveys.

To conclude, this thesis has provided a significant contribution to the current understanding on how oral biofilm-infections affect the general health. Even though several mechanistic explanations have emerged, we have provided novel information on endotoxemia and immunologic burden against periodontal pathogens as potential mediators of effect (**Figure 11**). Our findings further emphasize that all oral infectious diseases should be regarded as risk factors for cardiometabolic disorders, and ultimately for a shorter lifespan. Dentists should provide this information to patients by routine, and oral infectious foci should be thoroughly evaluated, especially in patients with other cardiometabolic risk factors. A blood test for periodontitis would be useful to develop, while it could be used in occupational health care as a matter of routine.

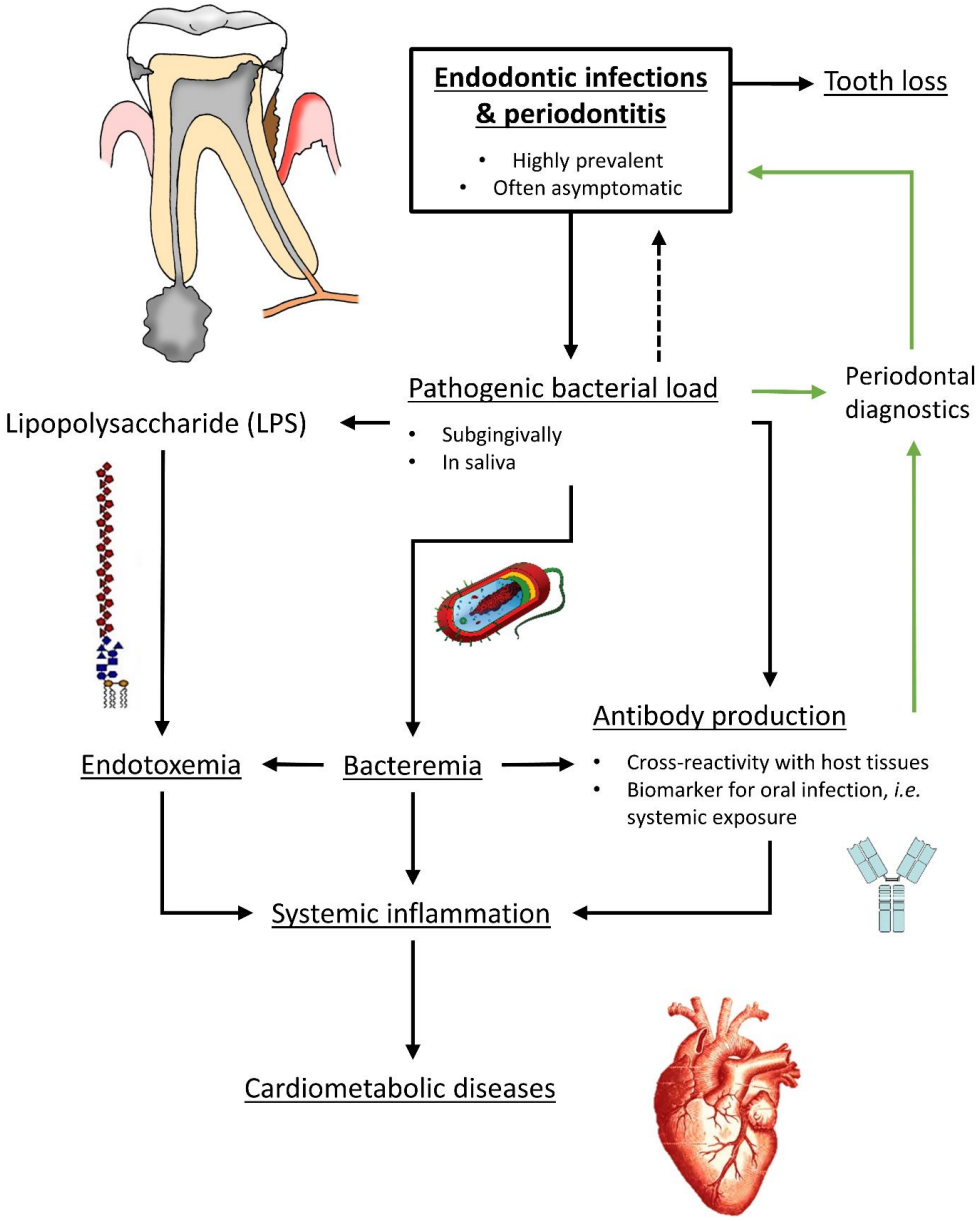

Figure 11. Schematic representation of how oral infections lead to a systemic exposure to bacteria, increasing the risk for cardiometabolic diseases.

Missing teeth represents a crude surrogate marker for a history of oral infectious diseases. A combination of oral bacterial levels and corresponding serum antibodies serve as a potential biomarker for periodontitis (green lines).

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